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## LAFAYETTE BENEDICT MENDEL

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With deep sorrow and a sense of irreparable loss the Journal records the death, on December 9, 1935, of Lafayette Benedict Mendel. His passing brings to a close the career of a distinguished scientist, an inspiring teacher, and a wise counselor.

Born in Delhi, New York, February 5, 1872, Mendel graduated from Yale in 1891. Two years later he received the Ph.D. degree from the same University. During the years 1895 and 1896, he continued his studies in physiology and physiological chemistry under Rudolf Heidenhain at the University of Breslau, and Eugene Baumann at the University of Freiburg.

For forty-three years Mendel was a member of the faculty of Yale. Beginning as an assistant in 1892, he gained early recognition by his remarkable aptitude for teaching and his success in research. In 1903 he was promoted to the rank of professor in the Sheffield Scientific School. From 1921 until the time of his death he served as Sterling Professor of Physiological Chemistry in Yale University. Although the author of more than three hundred publications, he is perhaps best known for his pioneer investigations upon vitamins and protein deficiencies. In collaboration with the late T. B. Osborne, Mendel was the first to demonstrate the nutritive significance of several dietary essentials. Many of his papers upon food "accessory factors" and the relation of amino acids to maintenance and growth are classics in the literature of nutrition.

Mendel was deeply interested in the Journal throughout the thirty years of its existence. He was a member of its first board of collaborators. In 1911, he became one of its editors, and continued in this capacity until his death. During this long period he gave generously of his time and energy in promoting the welfare of the Journal. His keen intellect and mature judgment rendered him an unusually valuable counselor. In no small measure was he responsible for the present status of the Journal as a successful

and enduring enterprise. His loyal and devoted labor in its behalf will not be forgotten.

As a man, Mendel possessed a personality of rare charm. He liked people, and his relation with them was characterized by a quiet dignity, courteous manner, and friendly interest. In the presence of his intimate friends he instinctively became the gracious host, entertaining those about him with his clever conversation and delightful humor. These personal attributes, together with his remarkable ability to impart to others the knowledge which he himself possessed, were responsible for his unusual versatility and resourcefulness as a teacher. Rare, indeed, are the men who have succeeded as he in firing the enthusiasm and inspiring the devotion of students. Because of these traits Mendel attracted to his laboratory, year after year, an unusually large group of young men and women. With kindly sympathy and understanding, and with infinite patience and tact he trained them in his and their chosen field of endeavor, and sent them out imbued with the ideals which he possessed and by which he lived. No wonder that many of his students of two or more decades ago still refer to him affectionately as "The Professor." On the occasion of the celebration of Mendel's sixtieth birthday, the late Graham Lusk said of him: "He has been the guide, philosopher, and friend to many young men and women; he has encouraged them to walk by themselves when they were able to stand alone; and he has given them wise counsel in times of difficulty. Herein he has shown himself as one of the great teachers of his time."

P. 17-1 A

## THE SERUM CALCIUM RESPONSE TO INGESTED CALCIUM

BY S. FREEMAN, E. R. KANT, AND A. C. IVY

*(From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago)*

(Received for publication, August 5, 1935)

Numerous studies have been made upon the serum calcium response following the ingestion of various calcium compounds. Kahn and Roe (1) reported large rises (80 per cent) in serum calcium following ingestion of 5 to 20 gm. of calcium lactate by fasting subjects. Bauer and Ropes (2) were unable to obtain such large rises, using 5 and 10 gm. doses of calcium lactate in normal human beings; they obtained an average maximal elevation of 8 per cent for the smaller dose and 14 per cent for the larger one. Liebermann (3) studied the absorption of calcium gluconate in human beings and found that 10 gm. doses produced an average maximal elevation of whole blood calcium in ten subjects from an original level around 12 mg. to 16.2 mg of CaO per 100 cc. Greenberg and Gunther (4) studied the influence of calcium lactate, gluconate, chloride, and acetate upon the serum calcium level of dogs. They concluded that these salts were all equally efficacious in raising the serum calcium and that the magnitude of elevation was proportional to the dosage of calcium. They do not state the definite dosage per kilo of body weight used in obtaining these data.

The present study, involving approximately 700 calcium determinations, was made upon normal adult persons and dogs and the study was primarily intended to answer two questions: (1) What effect, if any, does the daily ingestion of considerable doses of calcium have upon the serum calcium response to a given dose of calcium? (2) What is the relationship between serum calcium response and the dosage of calcium? Calcium chloride, gluconate, and lactate were used, and some comparative data were obtained on these salts. The dosage of calcium chloride given human sub-

## 2 Effect of Ca Ingestion on Serum Ca

jects was intentionally low because of the nauseating and irritating effects of large doses; also, because large doses are not frequently employed therapeutically for any length of time.

### EXPERIMENTAL

*Effect of Daily Ingestion of Calcium Chloride upon Serum Calcium Response to a Given Dose of This Compound*—It is generally conceded that the maximum serum response to peroral calcium occurs from 2 to 4 hours after ingestion (2-5). Kahn and Roe obtained a maximum elevation 4 to 7 hours after feeding calcium lactate. Our own experience on human subjects and dogs indicates that the maximal elevation of serum calcium occurs approximately 2 hours following administration of a calcium solution. The following results verify this point. (1) The average values on four human subjects given 2.3 gm. of calcium chloride orally are as follows:

	mg per 100 cc
Initial serum calcium level <sup>1</sup> . . . . .	11 1
Serum calcium concentration 2 hrs. later. . . . .	11 8
" " " 4 " " . . . . .	11 5

(2) The following are average serum calcium results on three dogs bled at hourly intervals after being fed 0.05 gm of calcium chloride per kilo of body weight by stomach tube.

*Serum Calcium, Mg. per 100 Cc.*

Initial	1 hr	2 hrs	3 hrs	4 hrs	5 hrs
10 6	11 5	12 1	11 6	11 3	11 0

As the maximal elevation was of primary interest, we made only two calcium determinations on each subject in the following series, before ingestion of 2.3 gm. of calcium chloride dissolved in 100 cc. of water and 2 hours after calcium ingestion. The eleven persons were all normal, each of an average weight of approximately 70 kilos, and in a fasting state when the determinations were made

<sup>1</sup> All calcium determinations were carried out in duplicate by the Clark-Collip procedure (6). No results are included in this paper whose duplicate determinations did not give titration values agreeing within 0.04 cc., and with very few exceptions the duplicates checked within 0.02 cc. of one another.

The same people continued daily ingestion of 2.3 gm. of calcium chloride for 1 month. The daily dose of calcium was taken in two portions, one before breakfast and the other before the evening meal. At the end of the month the serum calcium elevation was again determined following oral ingestion of 2.3 gm. of calcium chloride. The results are practically identical with those recorded for the same persons after one dose of calcium chloride and show neither a significant change in the initial calcium level nor in the magnitude of response to the dosage of calcium employed (Table I).

*Effect of Dosage and Anion upon Serum Calcium Elevation Following Oral Administration of Calcium Salts to Six Normal Adult Dogs*—Calcium lactate and gluconate were fed at the following

TABLE I  
*Effect of Single Dose of 2.3 Gm. of Calcium Chloride on Serum Calcium before and after 1 Month's Daily Ingestion of 2.3 Gm.*

	Before period of daily ingestion		After period of daily ingestion	
	Initial	2 hrs after ingestion	Initial	2 hrs after ingestion
Serum Ca, mg. per 100 cc .	11 0	11 6	10 8	11 4
Standard deviation, mg .	±0 38	±0 45	±0 46	±0 30
Coefficient of variation .	3 3	3 9	4 2	2 6
Maximum variation, mg	-0 8	-0 8	-0 7	-0 4
	to +0 4	to +0 9	to +0 3	to +0 6

levels of calcium administration per kilo of body weight: 0.05, 0.10, 0.15, 0.20, and 0.25 gm. Calcium chloride was administered in the following dosages: 0.05, 0.085, 0.10, and 0.15 gm.; larger doses of this salt were not included because of vomiting, which occurs much more frequently than at the higher levels of administration of the other two salts. The same six dogs, varying in weight from 12 to 22 kilos, were used for all of this series of experiments and were maintained on a uniform adequate diet for 2 months before and during the experiments. The animals were always in a fasting state when used. The serum calcium response was determined 2 hours after feeding. The dosage of calcium was always completely dissolved in 200 cc. of water and was fed by means of a stomach tube. The results of this study are summarized in Chart I

## Effect of Ca Ingestion on Serum Ca

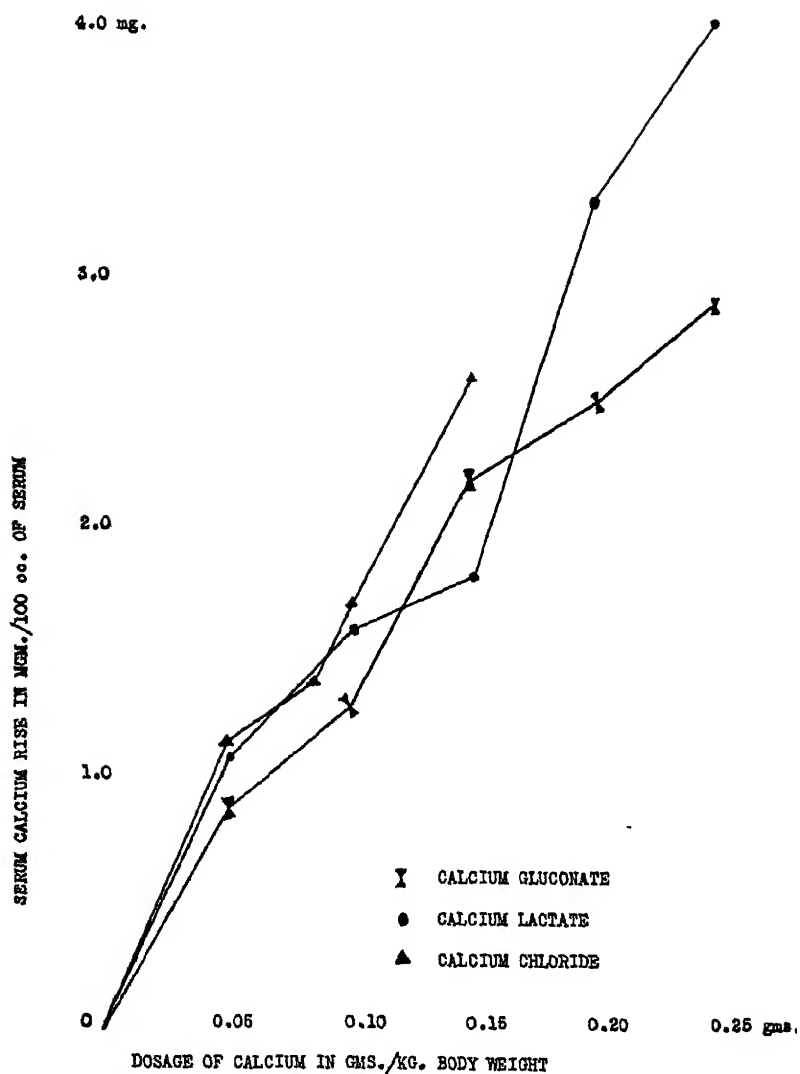


CHART I. Average serum calcium rise in six dogs, 2 hours after calcium administration.

in which the average serum calcium elevation for the six dogs is plotted against the level of calcium administration. The results show a progressive calcium increase as the dosage of calcium was elevated.

Calcium chloride and gluconate were also studied in another series of ten dogs. The dosage of calcium in this series was 0.05 gm. of calcium per kilo of body weight. The average serum calcium rise produced by each compound is given below. Calcium determinations were made before and 2, 4, and 6 hours after calcium administration.

*Serum Calcium, Mg per 100 Cc.*

	2 hrs	4 hrs	6 hrs
Calcium chloride	1 0	0 44	0 18
" gluconate	0 8	0 70	0 45

The rise obtained at the end of 2 hours is in good agreement, for both salts, with that obtained with the same salt and dosage in the series recorded in Chart I. Calcium chloride produces a somewhat greater maximal elevation of the serum calcium, but the duration of the elevation is greater for calcium gluconate.

The average serum calcium rise was also determined on four normal human subjects following ingestion, without breakfast, of 10 gm. of calcium gluconate dissolved in 200 cc. of water. The subjects were bled before and 2 and 4 hours after calcium ingestion. Average results, in mg. per 100 cc., are given below.

Initial	After calcium ingestion	
	2 hrs	4 hrs
10 5	11 0	10 7

These results show a serum calcium rise which is practically identical with that observed with a similar dosage of calcium chloride. The calcium dosage as calcium gluconate was 0.93 gm. and for calcium as calcium chloride had been 0.85 gm. The subjects fed calcium gluconate had also been used in the calcium chloride series.



## DISCUSSION

The results obtained upon human subjects show a slight but reasonably constant elevation of the serum calcium after ingestion of moderate doses of calcium chloride or gluconate. The doses of calcium which are given therapeutically probably produce no elevation of an initially normal serum calcium. Our results are in accord with the findings of Bauer and Ropes (2) as to the magnitude of serum calcium increase rather than with those obtained by Kahn and Roe (1) with calcium lactate or by Liebermann (3) with calcium gluconate. Either the daily ingestion of calcium chloride did not affect the saturation of the body tissues with calcium or else the repletion of the bone trabeculae with calcium has no influence upon the serum calcium response to ingested calcium.

The serum calcium response of normal fasting dogs can be predicted with a fair degree of accuracy if the dosage of calcium is known. Twenty determinations of the serum calcium elevation in normal dogs 2 hours after ingestion of 0.05 gm. of calcium as calcium chloride showed an average rise of 1.02 mg. per 100 cc. of serum; this value is in good agreement with the average obtained with this dose and compound included in Chart I. The average serum calcium rise obtained by Hjort (5) (who fed 0.19 gm. of calcium per kilo of body weight to dogs) 2 hours after calcium administration was 3.0 mg. per 100 cc. of serum. In Chart I our result after feeding 0.2 gm. of calcium as calcium lactate was an average rise of 3.3 mg. per 100 cc. Our data cannot be compared quantitatively with those given in Chart 5 of Greenberg and Gunther (4), as these workers do not express the dose of calcium in terms of body weight.

Hematocrit determinations, which were obtained along with the data shown in Chart I, indicate some dilution of the blood for the lower dosages of calcium, but, when the dose was 0.15 gm. per kilo or greater, there was no dilution of the blood and in many instances an actual concentration was indicated. The higher doses of calcium frequently produce diarrhea, which may not become apparent for several hours after calcium administration. It is possible that the higher dosages of calcium produce a part of their serum calcium increase by concentration of the blood.

Both calcium lactate and gluconate seemed well tolerated by

dogs; probably the latter was somewhat less irritating as judged by the frequency of diarrhea and the dosage level at which it occurred. The human subjects, with one exception, tolerated the daily dosage of calcium chloride without adverse manifestations.

#### SUMMARY

Daily ingestion of 2.3 gm. of calcium chloride for 1 month did not influence the serum calcium response of eleven normal adults to this amount of calcium. The serum calcium elevation 2 hours after administration to normal fasting persons of 2.3 gm. of calcium chloride or 10 gm. of calcium gluconate was between 5 and 6 per cent. The dosage of calcium influences the serum calcium response in fasted normal dogs over a range of calcium intake varying from 0.05 to 0.25 gm. of calcium per kilo of body weight. Calcium chloride produced a greater maximal serum calcium rise in ten normal dogs than did the corresponding dose of calcium gluconate, but the latter compound maintained a greater serum calcium rise 4 and 6 hours after ingestion.

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## A STUDY OF EQUILIN PREPARED FROM PREGNANT MARE URINE

BY GEORGE F. CARTLAND AND ROLAND K. MEYER

(From the Research Laboratories, The Upjohn Company, Kalamazoo)

(Received for publication, August 9, 1935)

Girard and coworkers (1) have reported that the crystalline theelin fraction obtained from the urine of the pregnant mare consists of a mixture of closely related substances which are separated with great difficulty. In addition to theelin, these workers have isolated and described three other estrogenic substances, namely: equilin, equilenin, and hippulin. From 7 tons of mare urine they isolated 100 mg. of equilin, m. p. 238–240° (corrected);  $[\alpha]_D^{15} = +308^\circ$  in dioxane; the benzoate, m. p. 195° (corrected). They report that equilin by the adult spayed rat method shows an estrogenic activity approximately one-seventh as great as theelin.

David and de Jongh (2) have recently reported that their preparations of equilin are one-third more active than theelin when tested by the spayed rat method. Dirscherl and Hanusch (3) have reported that equilin, which they obtained from Girard, assayed approximately equal to theelin when tested on spayed mice. These latter results are in marked disagreement with the relatively low biological activity reported by Girard.

We have prepared from mare urine a crystalline fraction which apparently is identical with the equilin described by Girard. Using late pregnancy urine, we have been able to obtain relatively much higher yields than those reported by Girard *et al* (1), thus making possible a further study of this fraction without the necessity of working up prohibitive quantities of mare urine.

*Preparation of Equilin*—24 gallons of mare urine collected during the last 2 months of pregnancy were acidified to Congo red with  $H_2SO_4$  and extracted twice with 0.5 volume of butyl alcohol. The combined butyl alcohol extracts were washed with 2 per cent  $Na_2CO_3$  and water. Further fractionation was carried out by the

method of Butenandt (4) with the modification that at his stage "Hormoncharge IV" crystallization from alcohol was substituted in place of high vacuum distillation. A crude crystalline material was obtained which after one recrystallization from ethyl alcohol weighed 494 mg.; m. p. 211–215° (corrected). This crystalline material contains equilin, theelin, and probably other closely related crystalline substances. Its recrystallization is summarized in Table I.

Five recrystallizations from ethyl alcohol yielded 59 mg. of a crystalline fraction, m. p. 236.5–238° (corrected);  $[\alpha]_D^{25} = +315^\circ$  (in alcohol);  $[\alpha]_D^{25} = +306^\circ$  (in dioxane); the benzoate, m. p. 196–197° (corrected). This material is apparently identical with the equilin described by Girard (1). At each stage of purification listed

TABLE I  
*Fractionation of Crystalline Material to Yield Equilin*

No of crystallizations	Weight of fraction	M p, corrected	$[\alpha]_D^{25}$ in alcohol	Chromogenic equivalent in terms of theelin
	mg	°C	degrees	per cent
1	494	211 –225		80
2	343	216 –223	+256	55
3	119	225 –228	+280	33
4	77	231 –234	+303	18
5	59	236 5–238	+315	15

in Table I, colorimetric assays were made by a method previously described (5) and the chromogenic values expressed as per cent of international standard theelin. The more purified equilin fractions showed low chromogenic values, and the consequently larger samples required for comparison against 0.005 mg. of standard theelin produced a yellow discoloration, making necessary the reading of the solutions in a Lovibond tintometer.

It will be noted from Table I that the purification of the equilin fraction is accomplished by the progressive removal of theelin as determined colorimetrically. This is supported by the fact that crystalline theelin, m. p. 259–260° (corrected), was isolated from the mother liquors. Since the colorimetric value of 15 per cent theelin obtained after five recrystallizations showed no proof of being stabilized, the results are suggestive of the possibility that

the chromogenic activity of equilin may be due to theelin contamination.

In order to study this possibility an additional 50 mg. of equilin were prepared by reworking the mother liquors from the previous fractionation, thus making available 100 mg. for further purification. The usual methods for separating theelin from theelol, based upon their differential solubilities in dilute alcohol and benzene and in different strengths of alkali, were ineffective in accomplishing any appreciable separation of theelin from equilin. Also, our experience with acetone and ethyl acetate as solvents for recrystallizing equilin was less satisfactory than with alcohol. Consequently, the recrystallization of the combined equilin frac-

TABLE II  
*Further Recrystallization of Equilin*

No of crystallizations	Weight of fraction	M p , corrected	$[\alpha]_D^{25}$ in ethyl alcohol	Chromogenic equivalent in terms of theelin
	mg	°C	degrees	per cent
	100	232-237	310	17
1	77	235-237		14
2	62	237-239		12
3	51	237-239	318	11
4	38	237-239	324	10
5	28	238-240	331*	9
6	20	238-240		11

\*  $[\alpha]_{5461}^{25} = +397^\circ$  in ethyl alcohol

tions was carried out with ethyl alcohol. The quantitative manipulation of these small amounts of material was made possible by crystallizing in 15 cc. Pyrex centrifuge tubes and using Jena fritted glass filter sticks for all filtrations. The results are given in Table II.

The results given in Table II indicate that a further purification of equilin has been accomplished by the removal of theelin contamination, as determined colorimetrically. The final product described in Table II was analyzed by microcombustion.

*Analysis*— $C_{13}H_{20}O_2$ . Calculated. C 80.55, H 7.52  
Found. " 80.51, " 7.76

Biological assay in sprayed rats by a method based on that of Kahnt and Doisy (6) and injection of both equilin and inter-

national standard theelin in aqueous 10 per cent alcohol containing 0.5 per cent  $\text{Na}_2\text{CO}_3$  into parallel groups of rats gave the following values: for equilin, 1 rat unit = 0.00087 mg.; for standard theelin, 1 rat unit = 0.00077 mg.; calculated, equilin contains 8850 international units per mg.

The chromogenic activity showed no significant change during the last four recrystallizations, which would indicate that the residual chromogenic activity is a property of equilin itself and is not due to the presence of approximately 10 per cent of theelin. It would have been desirable to recrystallize from different solvents at this point; however, the amount of material was too small to permit this procedure satisfactorily. Consequently, further information regarding the significance of the residual chromogenic activity of the purified equilin was obtained as follows:

The mother liquors from the crystalline fractions listed in Table II were submitted to a great number of recrystallizations, yielding 23 mg. of equilin, m. p. 238–240° (corrected),  $[\alpha]_D^{25} = +325^\circ$  in ethyl alcohol; theelin equivalent (colorimetric) 12 per cent. A mixed melting point with the final equilin fraction described in Table II was not depressed. Consequently 21 mg of the former and 8 mg. of the latter were joined, making 29 mg of equilin of a purity equal to the final product described in Table II. Injected in aqueous 10 per cent alcohol containing 0.5 per cent  $\text{Na}_2\text{CO}_3$  a value of 7120 international units per mg. was obtained.

This combined specimen of equilin was crystallized from alcohol, yielding three fractions, and each fraction was assayed biologically (6) and colorimetrically (5) with the following results:

6 mg of Crop 1    Biological assay, 7000 international units per mg.  
Chromogenic equivalent, 12 per cent theelin.

12 mg of Crop 2    Biological assay, 7400 international units per mg  
Chromogenic equivalent, 11 per cent theelin

10 mg. of mother liquor    Biological assay, 7700 international units per mg  
Chromogenic equivalent, 11 per cent theelin

Crop 2, obtained in this final fractionation, consisted of large crystals from which a single crystal weighing 0.617 mg. was isolated. Measurement of its principal angles gave values agreeing closely with those given by Girard (1) for his equilin. This crystal was weighed on the microbalance and dissolved in alcohol for biological and colorimetric assay. Biological assay, 7000 inter-

national units per mg. Chromogenic equivalent, 12 per cent theelin.

All of these final fractions, including the single isolated crystal, give, by colorimetric and biological assay, values which agree closely with those obtained for the purified equilin fraction obtained as described in Table II. This indicates that these specimens of equilin are pure and that the residual chromogenic value equivalent to approximately 11 per cent of theelin is a property of equilin itself and is not due to theelin contamination.

In all of the biological assays reported above equilin and the international standard theelin were injected in aqueous 10 per cent alcohol containing 0.5 per cent  $\text{Na}_2\text{CO}_3$ . The results indicate that equilin is approximately 75 per cent as active as theelin in the sprayed rat. On the other hand, by the colorimetric assay (5) equilin is only approximately 11 per cent as active as theelin. Thus, the colorimetric method is not very satisfactory for assaying equilin because of the discoloration produced by the relatively large samples required to give a red color equivalent to 0.005 mg. of theelin.

In a series of experiments in which equilin was injected in aqueous 10 per cent alcohol without the addition of 0.5 per cent  $\text{Na}_2\text{CO}_3$ , it was found that equilin is less than half as active as when administered in a similar solution containing 0.5 per cent  $\text{Na}_2\text{CO}_3$ . In this case a potency of 3000 international units per mg. was observed. The international standard theelin was injected, with the same solvent. This activity, equivalent to 30 per cent of that of standard theelin, approaches the low activity reported for equilin by Girard *et al.* (1). The higher potency, equivalent to 75 per cent of that of standard theelin, which we observed when equilin was injected in a solution containing 0.5 per cent  $\text{Na}_2\text{CO}_3$ , closely approximates the high biological potency observed by Dirscherl and Hanusch (3).

#### SUMMARY

From pregnant mare urine, equilin has been prepared which is apparently identical with that described by Girard (1).

Further purification of this material has accomplished the removal of a small amount of theelin which resulted in the isolation of equilin having a slightly higher specific rotation than that re-



ported by Girard. Evidence for purity is offered by comparative studies on a single isolated crystal of equilin.

Biological assays in spayed rats by a method based on that of Kahnt and Doisy (6) show that equilin is approximately 75 per cent as active as standard theelin when injected in aqueous 10 per cent alcohol containing 0.5 per cent  $\text{Na}_2\text{CO}_3$ . When injected without the addition of 0.5 per cent  $\text{Na}_2\text{CO}_3$ , equilin is approximately 30 per cent as active as standard theelin.

By a colorimetric method of assay (5) equilin is approximately 11 per cent as active as standard theelin.

We wish to thank Mr. Harold Emerson for the analyses by microcombustion.

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## STUDIES ON KETOSIS

### VII. QUANTITATIVE STUDIES ON $\beta$ OXIDATION. GLYCOGEN FORMATION FROM VARIOUS FATTY ACIDS\*

BY HARRY J. DEUEL, JR., JOSEPH S. BUTTS, LOIS F. HALLMAN,  
AND CHARLES H. CUTLER

(From the Department of Biochemistry, University of Southern California  
School of Medicine, Los Angeles)

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In a former paper (1) it was demonstrated that the excretion of acetone bodies in the urine of fasting rats was identical after the feeding of isomolecular quantities of butyric and caproic acids with that after diacetic acid was administered. Caprylic acid gave rise to a greater output of ketone bodies than diacetic acid, which would seem to indicate that a certain amount of  $\delta$  oxidation takes place. On the other hand, the odd carbon-chained fatty acids, propionic, valeric, and heptonic acids,<sup>1</sup> produced only minimum quantities of acetone bodies in the urine. Therefore,  $\alpha$  oxidation could not have occurred when valeric or heptonic acid was administered or acetone bodies would have originated thereby.

In view of the fact that the breakdown of the even chained fatty acids into acetone bodies has been found to be a quantitative one, it is of interest to determine whether  $\beta$  oxidation occurs with the fatty acids having an odd number of carbon atoms, as well as whether such a change is a quantitative one. The fact that the fatty acids with an odd number of carbon atoms do not give rise to acetone bodies in appreciable amounts does not necessarily prove  $\beta$  oxidation, although one may draw such inferential deductions from the data.

\* A preliminary report of this work has been published (Deuel, H. J., Jr., Butts, J. S., and Hallman, L., *Proc. Soc. Exp. Biol. and Med.*, **32**, 1351 (1935)).

<sup>1</sup> We have since demonstrated that nonylic (pelargonic) acid is incapable of giving rise to acetone bodies in rats.

Ringer (2) first noted that propionic acid was quantitatively converted to glucose in a phlorhizinized dog. In later work (3) he demonstrated that valeric acid also caused the excretion of "extra" sugar, although the results were not quantitative. Small increases were also shown in the glucose elimination after the administration of heptonic acid, while butyric and caproic acids as well as formic acid gave negative results. Deuel and Milhorat (4) demonstrated that acetic acid was not convertible to glucose in phlorhizinized dogs. Pollack (5) found an increase in blood sugar of fasted rabbits after the intravenous injection of the sodium salts of propionic and valeric acids but not after the salts of acetic, butyric, acetoacetic, or *n*-caproic acids. The fact that negative results on blood glucose were obtained after the feeding of the even chained fatty acids while positive ones were found only with the odd chained compounds is good proof that the hyperglycemia did not arise because of glycogenolysis.

On the other hand, Eckstein (6) was able to demonstrate only a small formation of glycogen after the administration of sodium propionate to fasting rats, while the results on valeric acid, sodium valerate, sodium butyrate, and sodium caproate were entirely negative

#### EXPERIMENTAL

##### *General Procedure*

Male and female rats from our stock colony of 75 to 120 days of age were used. The animals were fasted 48 hours, at which time the substance to be tested was fed by stomach tube. The lower fatty acids were administered as the sodium salts in a dose of 0.173 mole, equivalent to 10 gm. of acetone per sq. m. of body surface. Oleic acid was fed in a similar molar concentration as the free acid. Tributyrin and trivalerin were given in amounts of 19.5 and 22.2 gm. per sq. m., respectively, which gives an equivalent molar concentration of possible triose molecules (1 from glycerol and 3 from the fatty acids) as employed in the above tests. In the control experiments a similar dose of hydrolyzed tributyrin or glycerol as contained in the earlier tests was employed.

The animals were killed at several periods after the administration of the fatty acids. Amytal was used as an anesthetic. The technique for glycogen and fat determinations on the liver is

described elsewhere (7). The degree of absorption was determined by washing the small intestine and stomach, which had been removed intact, with water heated to 75° under sufficient pressure so that the intestines were dilated. About 50 to 60 cc. were used each time. After acidification of the washings with  $H_2SO_4$ , the solution was extracted four times with ether. The ether was evaporated from the extracted fat and the residue allowed to dry in a warm oven for several days. It was then transferred by dissolving in ether to weighed flasks and the fatty residue determined after removal of the solvent. That oleic acid and Wesson

TABLE I  
*Recovery of Fat from Water Suspension and from Intact Gastrointestinal Tract*

Substance	Procedure	Amount used	Amount recovered	
		gm	gm	per cent
Oleic acid	Recovery from water suspension*	0.894	0.842	94
			0.848	95
	“ “ “ “ †	0.894	0.910	102
			0.870	97
	“ after introduction into gastrointestinal tract†	0.894	0.868	97
Wesson oil	Recovery from water suspension*	0.920	0.877	98
			0.881	96
			0.852	93

\* Pipette used.

† Material measured with a 1 cc. tuberculin syringe

oil could be satisfactorily recovered by this procedure from the gastrointestinal tract is indicated in Table I.

The fatty acids used in these tests were Eastman products, the purity of which was established by titration.<sup>2</sup> The purity of the tributyrin and trivalerin was ascertained by the saponification number.

<sup>2</sup> Great difficulty was experienced in securing a satisfactory sample of valeric acid. The Eastman product showed only 78 per cent valeric acid equivalent by titration, but after several refractionations this value was increased to 92 per cent. We wish to express our thanks to Professor M. S. Dunn, of the University of California at Los Angeles, for the fractionations.

In the experiments in which glycogen formation is evident, the significance of the averages is ascertained by calculation of the ratio between the mean difference and the probable error of mean difference. When this exceeds 3, the results are considered significant.

### Results

The summary of the results for liver glycogen and fat of male rats 6 to 7 hours after receiving 0.173 mole of various fatty acids as sodium salts is given in Table II.

TABLE II  
*Liver Glycogen of Fasting Male Rats 6 to 7 Hours after Administration of Sodium Salts of Fatty Acids in Doses of 0.173 Mole*

Fatty acid fed (as Na salt)	No of rats	Average weight	Liver glycogen			Liver fat
			Mean	M D	Above control	
				P E (M D)*		
		gm	per cent		per cent	per cent
Controls . . . . .	19	216	0 23			3 80†
Odd chained						
Propionic . . . . .	9	229	1 36	8 70	88	4 43
Valeric . . . . .	20	205	0 67	10 00	100	3 98†
Heptole. . . . .	10	168	1 02	17 95	100	
Nonylic. . . . .	10	173	0 83	7 80	100	
Even chained						
Diacetic . . . . .	10	220	0 18			4 52
Butyric . . . . .	9	217	0 30			3 98
Caproic . . . . .	10	244	0 16			4 73
Caprylic . . . . .	10	174	0 23			

\* Ratio of mean deviation to probable error of mean deviation from control.

† Experiments on only ten animals.

In Table III are recorded the average results after the administration of tributyrin, trivalerin, Wesson oil, oleic acid, and the hydrolytic products of tributyrin.

### DISCUSSION

The data recorded here indicate that a profound difference exists in the glycogenic ability of odd and even chained fatty

acids. When isomolecular amounts of fatty acids belonging to the former category were fed, such as propionic, valeric, heptonic, and nonylic acids, significant amounts of glycogen were deposited in the liver; after the administration of similar quantities of the

TABLE III

*Liver Glycogen of Female Rats Previously Fasted 48 Hours at Various Periods after Administration of Various Fats and Their Derivatives*

Substance fed	Dose	Absorption time	No of rats	Average weight	Liver glycogen		Fat in gastro-intestinal tract	
						M D P E (M D)*		Absorbed
	mg per 100 sq cm	hrs		gm	per cent		mg	per cent
Control			26	162	0 10		4 6 (12)	
Tributyrin	195	8	13	177	1 22	19 64	6 3 (6)	
Trivalerin	222	8	10	157	1 85	18 05†	12 8 (3)	99 5
Wesson oil	195	8	10	183	0 05		12 3 (5)	98 6
“ “	761	8	10	153	0 05		261 9 (3)	86 4
Oleic acid	786	8	10	147	0 20		657 4 (4)	61 9
“ “	786	17	10	155	0 18		807 5 (8)	60 3
Glycerol	59	8	10	175	0 41	7 39		
Sodium butyrate	170	8	10	175	0 41	7 39		
Hydrolyzed tributyrin.	195†	8	10	159	0 61	7 95		
Glycerol..	59	8	10	177	0 36	2 82		
“	59	4	10	148	0 65	10 00		

\* Ratio of mean deviation to probable error of mean deviation from control

† The ratio of M D to P E. of M D between tributyrin and trivalerin animals is 5 73

‡ Dosage calculated on the basis of the original tributyrin.

acids of the second class, as diacetic, butyric, caproic, and caprylic acids, no rise in liver glycogen above the control level was noted

The formation of glycogen from the fatty acids with an odd number of carbon atoms is proof that the process of  $\beta$  oxidation applies to such acids. The simplest conception is that the fatty acids with 5, 7, or 9 carbon atoms must be degraded to propionic acid which serves as a building stone for glucose or glycogen.

That such a change is approximately a quantitative one is indicated by the fact that the amount of glycogen deposited falls within the same range in all cases. Although it was possible to demonstrate that the  $\beta$  oxidation occurred quantitatively in the change of the even chained fatty acids to the acetone bodies, it is more difficult to state that the transformation of the odd chained ones into glycogen takes place with as much exactness. One may conclude, however, that  $\beta$  oxidation occurs with such acids and that such a breakdown appears to be approximately a quantitative one.

The findings of Eckstein (6) on glycogenesis with valeric acid and his low results on propionic acid may possibly be due to the short interval which he allowed between the time of feeding and killing the rats. In the tests on valeric acid only 3 hours were allowed to elapse, while the glycogen determinations in our experiments were made following an interval of 6 to 7 hours. That a longer interval may be necessary for the formation of glycogen after the longer chained fatty acids than after propionic acid is indicated by some unpublished experiments in which it was noted that a rise in liver glycogen had already occurred 2 hours after propionic acid, while it was not detected until 4 hours after heptonic acid. A second factor which might be responsible for some of the negative findings of Eckstein is the size of the dose employed. Shapiro (8) noted that, while the introduction of lactic acid in a concentration of 1 mg. per sq.cm. was very satisfactory for glycogenesis, the administration of 3 mg. per sq.cm. was definitely toxic and no glycogen synthesis was noted. In some of our experiments, also unpublished, it was found that glycogenesis could not be demonstrated consistently when the doses of the acids were increased from 1 to 1.5 mg. per sq.cm. of body surface (calculated as acetone). It is probable that 800 mg. of valeric acid which Eckstein employed in some cases would be toxic. At the level of 1 mg. per sq. cm. used by us a 150 gm. rat would receive only 450 mg. of valeric acid.

Further confirmation of the glycogenic ability of the odd chained fatty acids is afforded by the higher glycogen formation after trivalerin than was obtained after tributyrin. In the former case, the mean glycogen level amounted to 1.85 per cent as compared to one of 1.22 per cent after tributyrin.

The high level of glycogen formation after tributyrin as compared with a control value of 0.10 per cent is difficult to explain. Shapiro (8) obtained a level of 1.91 per cent of liver glycogen 4 hours after feeding glycerol in approximately 5 times the dose given as a constituent of the neutral fat here. With amounts of glycerol (0.59 mg per sq cm) comparable to those employed in the present tests, the glycogen deposition after 8 hours was only 0.36 per cent, but after only 4 hours it amounted to 0.65 per cent; when mixed with the equivalent amount of sodium butyrate which would originate from the hydrolysis of tributyrin, the glycogen level was 0.41 per cent. Only slightly higher results (0.61 per cent) were noted after the administration of saponified tributyrin. Whether this indicates that tributyrin gives rise to more sugar than is produced by the glycerol equivalent is not a matter of importance in the present communication. The rate of absorption and glycogen formation when glycerol is fed as such or as a component of neutral fat must be quite different. The results on butyric acid alone also indicate that it is not to be considered as a glycogenic agent.

That the non-glycerol portion of the physiological fats is not a source of glucose is indicated by the failure of Wesson oil to increase the level of liver glycogen. Not only when this fat was fed in the same dose as tributyrin (195 mg.) but even when fed in a molecular equivalent (761 mg.), we have failed to note an appreciable glycogen formation. Although the absorption of the fat was somewhat lower in the test with the larger doses than the complete absorption noted for tributyrin, 86 per cent disappeared from the gastrointestinal tract.

The failure of such a neutral fat as Wesson oil to give rise to glycogen while tributyrin in isomolecular amounts is able to cause an appreciable glycogen deposition may be explained in the following way. According to Eckstein (9) butyric acid cannot be stored as such in the body. Since this is the case, the glycerol in tributyrin cannot be stored away as a component of neutral fat and it is therefore converted to glycogen. On the other hand, the triolein, tripalmitin, and tristearin, of which Wesson oil is chiefly composed, which are normal components of storage fat of rats, can be deposited as such and the glycerol is not free to be converted to glucose.



The results with oleic acid also are quite definite in demonstrating no appreciable glycogenic action after 8 or 17 hours. We had supposed that if the cleavage of oleic acid at the double bond to 2 molecules of nonylic acid occurred, the 9-carbon fatty acid so formed would give rise to appreciable quantities of glycogen. The negative results indicate that such a breakdown is probably of small significance. However, considerable amounts of the acid remained unabsorbed after 17 hours and an undetermined amount was lost by diarrhea. An attempt to overcome the obstacle of absorption by intraperitoneal injection of oleic acid failed because of its toxicity.

#### SUMMARY

1. No significant glycogen deposition occurred in the livers of fasting male rats following the administration of sodium acetoacetate, sodium butyrate, sodium caproate, sodium caprylate, or oleic acid.

2. Glycogen deposition in the liver followed the administration of propionic, valeric, heptonic, and nonylic acids as the sodium salts in doses of 0.173 mole per sq.m. of body surface. Inasmuch as the level of liver glycogen is in the same range, it is concluded that  $\beta$  oxidation proceeds in a relatively quantitative manner with fatty acids having an odd number of carbon atoms.

3. Trivalerin was found to be a significantly better glycogenic agent than tributyrin. No glycogen formation was noted after the administration of Wesson oil in similar molecular quantities.

4. The formation of glycogen after tributyrin is largely, if not entirely, to be traced to its glycerol component. However, after the administration of similar amounts of glycerol, glycerol plus sodium butyrate, or saponified tributyrin only part of the glycogen deposition could be accounted for. It seems possible that differences in time relationship in glycogen formation due to the effect of the fats on the rate of absorption may account for these variations.

5. The fact that oleic acid does not bring about significant glycogen formation, while nonylic acid does, must indicate that cleavage at the double bond into 2 molecules of the latter acid cannot be a primary change in the metabolism of oleic acid.

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## THE PEPTIDASE SYSTEM OF *ASPERGILLUS* *PARASITICUS*\*

BY MARVIN J. JOHNSON AND W. H. PETERSON

(From the Departments of Agricultural Chemistry and Agricultural Bacteriology, University of Wisconsin, Madison)

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Within the last decade, the enzymes classically known as erepsins have been shown to be rather complex enzymic systems whose single components have been named peptidases. Owing to the work of Waldschmidt-Leitz (1), Abderhalden (2), Grassmann (3), and their respective coworkers, there exists at least an approximate knowledge of the properties of the individual peptidases of two naturally occurring peptidase systems, that of the mammalian intestinal tract and that of yeast.

The object of the present investigation has been to study the structure of the proteolytic system of a typical mold, in order to determine to what extent it resembles known proteolytic systems. As reported in a previous paper (4), the proteolytic system of *Aspergillus parasiticus* was found to consist of at least four components. Besides a proteinase, three peptidases were found which seemed to have properties resembling those of the aminopolypeptidase, the carboxypolypeptidase, and the dipeptidase, respectively, of the animal digestive enzyme system.

### EXPERIMENTAL

#### *Methods*

In general, the experimental procedure was similar to that described previously. Aqueous extract of fresh *Aspergillus parasiticus* tissue was used as a source of enzyme material. The mold was grown at 30° on a medium consisting of skim milk to which 5 per cent of glucose was added. The water extraction was carried out at pH 7.

\* This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

The methods of enzyme determination, summarized in Table I, are similar to those previously used (4). For the peptidase determinations, a stock substrate solution was prepared such that if 4 cc. of it were diluted to 6 cc. with enzyme and water, the substrate and buffer concentrations would be those given in Table I. This enzyme-substrate mixture was then incubated for a definite time, usually 1 hour.

TABLE I  
*Methods of Enzyme Determination*

Enzyme	Substrate	Substrate concentration	pH	Buffer (M/15)	Relation between degree of hydrolysis and amount of enzyme
Carboxy-polypeptidase	Chloroacetyl-L-tyrosine	M/30	7.0	Phosphate	$E = \frac{1}{t} \log_{10} \frac{1}{1-x}$
Aminopolypeptidase	dl-Leucylglycine	M/15	8.0	Borate	Same
Dipeptidase	dl-Leucylglycine	M/15	7.25	Phosphate	$E = 0.602x^*/t$
Proteinase	Gelatin†	4%	7.25	"	Curve‡

6 cc. of reaction mixture Incubation at 40°, usually for 60 minutes.

$E$  = the number of enzyme units present in reaction mixture.

$t$  = incubation time in minutes.

$x$  = fraction of  $l$  form of peptide hydrolyzed during incubation

\* The factor 0.602 is introduced in order to make the size of the dipeptidase unit such that half hydrolysis of the substrate corresponds in all three determinations to the same number of units.

† 10 cc. of reaction mixture.

‡ The number of enzyme units present is obtained by comparison with an experimentally determined empirical hydrolysis curve (4).

For the amino nitrogen estimations before and after incubation, it was found that the Van Slyke method could be advantageously replaced by the Landerström-Lang acetone titration method (5). 2 cc. aliquots of the enzyme-substrate mixture were titrated in 90 per cent acetone with M/15 alcoholic HCl.

The formula relating degree of hydrolysis and number of enzyme units present, based, of course, on the kinetics of the hydrolysis process under the conditions of the determination, varies with the

enzyme being determined. While rates of hydrolysis of leucyl-diglycine and of chloroacetyltyrosine are proportional to substrate concentration, the rate of leucylglycine hydrolysis is independent of substrate concentration (4).

An incubation temperature of 40° was chosen, since this temperature is the highest at which the destruction of enzyme during a 1 to 3 hour incubation period is inappreciable. During longer incubation periods, however, there is very considerable loss of enzyme. Heating at 40° for 14 hours at pH 7.2 results in the destruction of as much as 30 per cent of the enzyme.

*Synthetic Substrates*—The peptides used in the present investigation were prepared through the corresponding halogenacyl compounds by the methods of Fischer and coworkers (6). The N-methyl peptides were prepared by analogous methods, by the use of methylamine instead of ammonia (7). The substituted amides (leucylmethylamine and sarcosylmethylamine) were prepared by the method of von Braun and Munsch (8). The purity of the peptides and other synthetic substrates used was checked by determinations of neutral equivalent by one or more of three methods: titration of the carboxyl group in 90 per cent alcohol with thymolphthalein indicator, the Linderstrøm-Lang amino titration (5), and determination of the chlorine content of the hydrochloride. The authors wish to express their thanks to Dr. L. H. Andrews for the preparation of a number of these compounds.

*Purification of Enzyme Material*—The crude enzyme-bearing material, made by aqueous extraction at pH 7 of the mold tissue, may be very considerably purified, without great loss of enzyme, by precipitation of the enzyme from the extract with an equal volume of acetone. The resulting precipitate is centrifuged off and redissolved in water. Purification by this method results in a 5-fold increase in the purity of the preparation. The yield of enzyme is from 65 to 100 per cent of that present in the raw material.

If such an acetone-precipitated preparation is ultrafiltered through a cellophane membrane (du Pont No. 300, plain transparent) at from 2 to 10 atmospheres pressure, the enzymes do not pass the membrane, but a large amount of impurity does. This procedure increases the purity of the preparation to approximately 30 times that of the original aqueous mold extract.

*Purification of Aminopolypeptidase*—The method previously described for the preparation of purified aminopolypeptidase has been somewhat improved. It has been found that if a solution containing aminopolypeptidase and dipeptidase is treated with from 1.5 to 2.5 volumes of 95 per cent alcohol, the aminopolypeptidase is precipitated to a greater extent than the dipeptidase. The quantity of alcohol necessary to bring about the best separation varies with different preparations. An example of the purification process as applied to one sample follows.

To 100 cc. of a solution which had been purified by acetone precipitation was added acetic acid to pH 4. The resulting precipitate was centrifuged off. Much of the dipeptidase is thus removed. After adjustment to pH 7, the solution was treated with 250 cc. of 95 per cent alcohol. The precipitate was centrifuged off and dissolved in 50 cc. of water. To this solution were added 80 cc. of alcohol and about 0.1 gm. of sodium acetate to assist flocculation. The precipitate was centrifuged off and dissolved in 50 cc. of water. The solution was adjusted to pH 4 with acetic acid, and adsorbed with 10 mg. of  $\text{Al}(\text{OH})_3$  C $\gamma$ , followed by 20 mg. of  $\text{Fe}(\text{OH})_3$ . The resulting preparation contained 32 per cent of the aminopolypeptidase present in the crude material, and had an aminopolypeptidase to dipeptidase ratio of 104.

*Properties of Mold Aminopolypeptidase*—In Table II are shown the relative speeds of hydrolysis of various compounds by purified mold aminopolypeptidase. The enzyme, as may be seen, has the properties of a conventional aminopolypeptidase. Methyl substitution on the free amino group prevents enzyme action. As is the case with yeast aminopolypeptidase, the decarboxylated dipeptide, *dl*-leucylmethylamine, is hydrolyzed, apparently owing to the fact that the carboxyl group, whose proximity to the linkage to be split prevents hydrolysis of dipeptides, is here missing and therefore its effect on the basicity of the peptide nitrogen atom is not felt. It might be assumed that leucylmethylamine was hydrolyzed by the trace of dipeptidase present. That such was not the case was shown by subjecting this substrate to the action of a dipeptidase-rich enzyme preparation. It was hydrolyzed even less rapidly than by the purified aminopolypeptidase (see Table IV).

As was previously reported, triglycine is not hydrolyzed by

mold aminopolypeptidase, although it is readily attacked by yeast or intestinal aminopolypeptidase. It was thought that the extreme resistance to hydrolysis of triglycine, as compared with leucyldiglycine, could perhaps be due to a difference in basicity of the amino groups of the two compounds. Accordingly, the appar-

TABLE II  
*Hydrolysis of Peptides by Purified Aminopolypeptidase*  
M/15 substrate at pH 8 (M/15 borate), 40°.

Substrate	Hydrolysis* after		
	1 hr	4 hrs	10 hrs
	per cent	percent	per cent
<i>dl</i> -Leucyldiglycine . . .	33	45 5	47 5
<i>dl</i> -Leucylglycine . . .	1 0	4 0	11 0
Triglycine . . .	-0 5	-0 5	+0 5
Glycyl- <i>dl</i> -leucylglycine	6 5	16 5	27 5
Benzoyldiglycine . . .	-0 5	-0 5	+0 5
<i>dl</i> -N-Methyllaucyldiglycine . .	0	0	0
<i>dl</i> -Leucylmethylamine . . .	1	4	11 5

\* Calculated as per cent hydrolysis of one linkage of peptide, including both components of racemic peptides

TABLE III  
*Apparent Basic Dissociation Constants at 25°*

Compound	pH of half neutralized 0.2 M solution	$K_b \times 10^{12}$
<i>dl</i> -Leucine . . . . .	2.36	2.3
<i>dl</i> -Leucylglycine . . . . .	3.19	16.0
<i>dl</i> -Leucyldiglycine . . . . .	3.27	19.0
Glycine . . . . .	2.40	2.5
Diglycine . . . . .	3.16	14.0
Triglycine . . . . .	3.27	19.0

ent basic dissociation constants of the two peptides, as well as those of leucine, glycine, leucylglycine, and diglycine, were determined. The method used was the determination of the hydrogen ion concentration, by the glass electrode, of a solution 0.2 M with respect to the peptide and 0.1 M with respect to HCl. The results obtained are shown in Table III. The values for leucine and



glycine check those obtained by Harris (9). The constants for leucyldiglycine and for triglycine were found to be the same. The difference in behavior of the two compounds toward the enzyme is therefore not due to a difference in basicity of the free amino groups. It was also found that neither triglycine, nor N-methylleucyldiglycine, inhibited leucyldiglycine hydrolysis by aminopolypeptidase; therefore, neither compound combines with the enzyme. The N-methylleucyldiglycine does not combine with the enzyme because it lacks the essential free amino group, but the reason for the inertness of triglycine is not clear. It cannot be due to a lack of steric adaptation of the enzyme for the glycyl radical, since glycylleucylglycine is readily hydrolyzed.

It was previously reported (4) that mold aminopolypeptidase apparently attacked both components of *dl*-leucyldiglycine. This conclusion has been found to be erroneous. The diglycine produced as a product of leucyldiglycine hydrolysis is partly decomposed during the Van Slyke determination, giving rise to fictitiously high  $\text{NH}_2\text{-N}$  values. When leucyldiglycine hydrolysis is followed by means of the acetone titration method, it becomes evident that only one component of the racemic peptide is split. In Fig 1 the course of leucyldiglycine hydrolysis by purified aminopolypeptidase is shown. It will be seen that hydrolysis does not exceed 50 per cent. Also in Fig. 1 the course of *dl*-leucylglycine hydrolysis by crude mold enzyme is shown. The hydrolysis rate is constant up to approximately 35 per cent hydrolysis, but then falls off, never exceeding 50 per cent.

*Other Mold Peptidases*—As was previously reported, the mold enzyme complex contains, besides aminopolypeptidase, at least two other peptidases. One, attacking leucylglycine, was called a dipeptidase, and the other, splitting chloroacetyltyrosine, was called carboxypolypeptidase. Neither of these was obtained in a purified state, and nothing was learned regarding their properties beyond the fact that neither would attack the specific substrate for the other. It was thought that any attempt to purify one of these peptidases should be preceded by a study of the hydrolytic properties of the peptidase complex as a whole. Such a study should give an indication of the probable number of peptidases, and of some of the properties of each. A number of synthetic peptides, none of which is attacked by purified aminopolypepti-

dase, were subjected to the action of crude mold enzyme. Data are given in Tables IV and V. The data of these tables are strongly suggestive of a general rule to the effect that benzoilation of the amino group of a glycyl peptide prevents hydrolysis, whereas methylation, or substitution of a chlorine atom for the amino group, does not. If hydrolysis of the sarcosyl compounds is due to a carboxypolypeptidase, this enzyme must have the properties described for the carboxypolypeptidase enzyme by Abderhalden and Schwab (10), rather than the acylase-like properties described by Waldschmidt-Leitz and Purr (11).

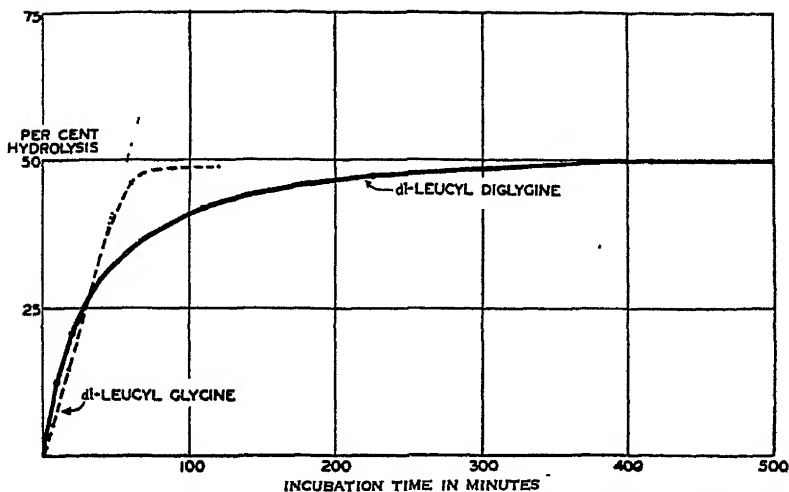


FIG 1. Course of hydrolysis of *dl*-leucylglycine and *dl*-leucyldiglycine by mold peptidases.

The ready hydrolysis of triglycine by the crude enzyme is apparently not due to the dipeptidase, since the ratio of dipeptidase to triglycine-hydrolyzing ability varies as much as 30-fold in different preparations. On the basis of present data, no definite conclusions can be drawn regarding the type of peptidase responsible for triglycine hydrolysis.

Inhibition experiments have shown that leucylglycine hydrolysis is strongly inhibited by the presence of diglycine (44 per cent inhibition by 0.133 *M* diglycine), indicating that the leucylglycine-hydrolyzing enzyme combines readily with diglycine. The

hydrolysis of diglycine itself is, however, at the substrate concentrations used, a first order reaction, indicating that only an inappre-

TABLE IV  
*Per Cent Hydrolysis of Compounds by Crude Mold Enzyme (Preparation 4)*

Substrate	Substrate concentration	pH	Hydrolysis* after		
			1 hr	3 hrs	18 hrs
Diglycine	M/15	8 0		10	39
Triglycine . . . . .	M/15	8 0		18	
Benzoyldiglycine . . . . .	M/15	8 0		0	0
<i>dl</i> -N-Methylleucyldiglycine . . . . .	M/15	8 0		1	0
<i>dl</i> -Leucylglycine . . . . .	M/15	7 2	45	48	
Chloroacetyl- <i>l</i> -tyrosine	M/30	7 0	22		
<i>dl</i> -Leucyldiglycine	M/15	8 0	32		
<i>dl</i> -Leucylmethylamine . . . . .	M/15	8 0		2 5	

\* On the same basis as in Table II

TABLE V  
*Hydrolysis of Compounds by Crude Mold Enzyme (Preparation 9)*

Substrate (M/30, pH 8 0)	Per cent of one linkage hydrolyzed after		
	1 hr	4 hrs	18 hrs
Triglycine . . . . .	11	45	81
Sarcosyldiglycine . . . . .	1	6	16
Benzoyltriglycine . . . . .	-1	-2	+1
Chloroacetyldiglycine . . . . .	2	3	5
Diglycine . . . . .	22	56	92
Sarcosylglycine . . . . .	0	9	21
Chloroacetyl-glycine . . . . .	4	16	36
Glycyl- <i>l</i> -tyrosine . . . . .	15	46	60
Sarcosyl- <i>l</i> -tyrosine . . . . .	3	8	22
Chloroacetyl- <i>l</i> -tyrosine . . . . .	37		
Chloroacetyl-glycine ethyl ester . . . . .	4*	12*	26*
Sarcosylmethylamine . . . . .	0	0	-1
Glycylsarcosine . . . . .	0	0	0
<i>dl</i> -Leucylglycine† . . . . .	48		

\* Ethyl alcohol determination showed that most of the substrate had saponified during incubation

† M/15 substrate concentration.

cialable percentage of the enzyme exists as enzyme-substrate complex. It follows that leucylglycine and diglycine must be

hydrolyzed by different enzymes. Additional evidence for such a conclusion is the fact that glycylsarcosine inhibits diglycine hydrolysis, but not leucylglycine hydrolysis.

Since the ratio of leucylglycine-hydrolyzing power to chloroacetyltyrosine-hydrolyzing power varies greatly (from 3.5 to 13), with different preparations, these two compounds are undoubtedly not hydrolyzed by the same enzyme. Therefore, unless all the chloroacetyl compounds, all the sarcosyl compounds, and all the polyglycines are hydrolyzed by the same enzyme, which does not at present seem probable, it is necessary to assume the presence of at least three peptidases in addition to the aminopolypeptidase. It is hoped that work now in progress will yield more definite information concerning the properties of these peptidases.

#### SUMMARY

1. An improved method for the preparation of mold aminopolypeptidase is described.

2. The aminopolypeptidase of *Aspergillus parasiticus* requires a free amino group as a point of attachment for the enzyme. It hydrolyzes the peptide linkage adjacent to this free amino group. Dipeptides are not attacked unless the inhibiting influence of the carboxyl group is removed by decarboxylation. Peptides composed entirely of glycine are not attacked.

3. The mold peptidase system contains enzymes capable of hydrolyzing peptides in which the free amino group is methylated, or replaced with a chlorine atom. Benzoyldiglycine and benzoyltriglycine are not hydrolyzed by the mold peptidase system.

4. Although the aminopolypeptidase of mold does not attack triglycine, and the leucylglycine-hydrolyzing dipeptidase of mold does not attack diglycine, the crude mold peptidase complex splits both of these peptides.

5. The apparent basic dissociation constants of leucyldiglycine, leucylglycine, triglycine, and diglycine have been determined.

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# STUDIES OF MULTIVALENT AMINO ACIDS AND PEPTIDES

## V. CYSTINE CYAMIDENE

By JESSE P GREENSTEIN

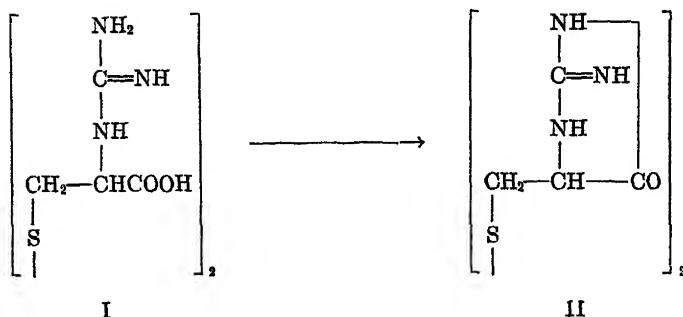
*(From the Department of Physical Chemistry, Harvard Medical School,  
Boston)*

(Received for publication, July 13, 1935)

Disulfide organic compounds as well as their reduced thiol derivatives play an important rôle in the biochemistry of all organisms. An almost equally vital part is contributed by substances containing the guanidine nucleus. Although no compounds containing both these types of groupings in the same molecule have been found among the products of biological origin, it would be of interest, nevertheless, to prepare such substances and to observe some of their physiological and physicochemical properties.

Diguanidocystine has been prepared by Kapfhammer and Muller (6) by the use of S-ethylisothiurea hydrobromide on the sodium salt of cystine. The author has described (5) the formation of an  $\epsilon, \epsilon'$ -diguanido-di( $\alpha$ -thio-*n*-caproic acid) by the use of the free O-methylisourea base on the corresponding amino acid. These are the only substances so far prepared containing both disulfide and guanidine groups on the same molecule. In the endeavor to extend the studies on guanidocystine there is described in this communication the preparation of the corresponding cyamidene derivative of cystine, namely anhydro- $\alpha, \alpha'$ -diguanido-di( $\beta$ -thiopropionic acid) as the hydrochloride, the picrate, and the free base. The action of alkali on the cyamidene is also described. As would be expected from the behavior of analogous substances, it is extremely labile to even very low concentrations of alkali.

## EXPERIMENTAL



Diguanidocystane (I) was prepared by the method of Kapfhammer and Muller (6) with the exception that the S-methylisothiourea base was used in place of the S-ethyl homologue which the above authors employed. The formation of the cyamidene (II) was easily accomplished by heating with concentrated HCl.

*$\alpha, \alpha'$ -Diguanido-Di( $\beta$ -Thiopropionic Acid)*—An amount of 19.88 gm. of pure cystine was dissolved in 145.3 cc. of 1.14 N NaOH. To the solution were added, with stirring, 23.03 gm. of S-methylisothiourea sulfate. The solution was then pumped off in a vacuum desiccator, the methyl mercaptan evolved being removed in about 5 hours. After 24 hours the crystals of diguanidocystine were filtered off and washed thoroughly with water, alcohol, and ether. The material was recrystallized from dilute HCl solution by the use of ammonia, and finally washed and dried. The yields were always somewhat lower than those which Kapfhammer and Muller (6) reported, the difference probably being due to the relatively greater instability of the S-methylisothiourea base as compared with the ethyl homologue. The substance crystallized in rectangular plates. The Sakaguchi reaction was positive. The diguanidocystine requires boiling with alkali before a blackening is obtained with lead acetate solution.

$\text{C}_5\text{H}_{12}\text{O}_4\text{N}_6\text{S}_2$  (324.13). Calculated, N 25.9; found, N 25.6

*Anhydro- $\alpha, \alpha'$ -Diguanido-Di( $\beta$ -Thiopropionic Acid) Dihydrochloride*—9 gm. of the diguanidocystine were dissolved in 180 cc. of concentrated HCl and evaporated down twice on the water bath to dryness. Complete drying was finally accomplished

*in vacuo*. The residue is a white solid, m.p.  $144^{\circ}$ , which was first washed with ethyl alcohol and then recrystallized from a hot methyl alcohol-ethyl alcohol mixture. The cyamidene derivative as the hydrochloride salt separates in the form of long prisms. It was washed with dry ether and dried. Yield 5 gm. The substance gave a negative Sakaguchi reaction, was soluble in water and hot methanol, but insoluble in all other organic solvents. M.p.  $150^{\circ}$  for the pure material.

The substance dissolved in dilute alkali, and when immediately treated with lead acetate solution gave an instantaneous blackening.

$C_8H_{14}O_2N_6Cl_2S_2$  (361.1) Calculated, N 23.2; found, N 22.8

*Anhydro- $\alpha$ , $\alpha'$ -Diguanido-Di( $\beta$ -Thiopropionic Acid)*—The free base was liberated from the hydrochloride by dissolving 3 gm. of the latter in a little cold water and adding ice-cold 3 N  $NH_4OH$  in slight excess. The base separates out in the form of tiny rectangular plates. It was filtered off, washed with water, alcohol, and ether, and dried *in vacuo* over  $P_2O_5$  and  $H_2SO_4$ . Yield 2.15 gm. It is extremely insoluble in all solvents save acids, and yielded a negative Sakaguchi reaction. The substance begins to turn dark on heating above  $240^{\circ}$ . The behavior in alkaline solution with lead was, of course, identical with that of the hydrochloride.

$C_8H_{12}O_2N_6S_2$  (290.1). Calculated, N 28.9; found, N 28.6

The picrate was formed by adding 0.72 gm. of the free base to a hot solution of 1.25 gm. of picric acid in 100 cc. of water. The yellow colored *half picrate* crystallizes in prisms. M.p.  $188^{\circ}$ . Yield 1.12 gm.

$C_8H_{12}O_2N_6S_2 \cdot C_6H_5(OH)(NO_2)_3$

Picric acid from nitron picrate. Calculated, 44.1; found, 43.8

*Action of Alkali on Cystine Cyamidene*—From the work of Bergmann and Stather (3) on dialanylecystine anhydride and of Andrews and Andrews (1) on cystine phenylhydantoin it is apparent that compounds of ring structure linked by disulfide bonds are extremely sensitive to alkali. The mechanism proposed by Bergmann and Dehs (2) for the decomposition of serine phenylhy-



dantoin, whereby the molecule first forms the 3-phenyl-5-methylenehydantoin, and then further splits to phenylurea and pyruvic acid, was found applicable also by Andrews and Andrews (1) to the case of cystine phenylhydantoin

The action of alkali on cystine cyamidene follows an analogous path. On mixing 2 gm. of the substance with 4.6 gm. of phenylhydrazine hydrochloride, 5 gm. of lead acetate, and 80 cc. of 1 N NaOH, there is an instantaneous blackening. The mixture is heated on the water bath for 12 hours, during which interval  $\text{NH}_3$  is evolved. The PbS is filtered off and the filtrate acidified with glacial acetic acid. The yellowish crystals of the phenylhydrazone of pyruvic acid separate out. Yield 1.2 gm. After recrystallization from alcohol its melting point is  $192^\circ$ , which is in agreement with the value found by Bergmann and Delis (2) and Clarke and Inouye (4).

The course of the reaction with alkali therefore involves the following steps: first, the breakdown of cystine cyamidene to 5-methylenecyamidene, or  $\alpha$ -aminoacrylic acid cyamidene and sodium sulfide; secondly, the hydrolysis to guanidine and pyruvic acid. The guanidine is destroyed during the reaction, accounting for the ammonia given off.

#### SUMMARY

1. Cystine cyamidene has been prepared in the form of the hydrochloride (m.p.  $150^\circ$ ), the half picrate (m.p.  $188^\circ$ ), and the free base (darkening above  $240^\circ$ ).

2. Like all ring compounds involving the disulfide linkage, it is extremely sensitive to alkali, decomposing very rapidly to alkali sulfide, pyruvic acid, and guanidine. The phenylhydrazone of pyruvic acid was isolated following alkaline treatment.

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## SOME OBSERVATIONS ON THE ISOLATION OF CYSTINE FROM WOOL HYDROLYSATES\*

BY GERRIT TOENNIES AND MARY A. BENNETT

(*From the Lankenau Hospital Research Institute, Philadelphia*)

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Methods proposed for obtaining cystine from wool, etc., vary widely with respect to the conditions used for hydrolysis and isolation (1-13). In many cases a negative biuret test is taken as a sign of completed hydrolysis. The best yields of cystine correspond to about two-thirds of the total sulfur content of the wool (7), although by the Sullivan test cystine corresponding to the total sulfur content has been identified after hydrolysis (14). However, the proportion of *l*-cystine obtained, presumably the only form of cystine occurring naturally, is not evident in most reports (*cf.* 15)).

It was in search for a reliable method for the complete isolation of cystine, more especially the levo form, that the present studies were undertaken, with a view of establishing a basis for the systematic study of the factors controlling hydrolytic liberation and racemization of cystine, a study necessary for the establishment of a rational method for the production of optimal yields of *l*-cystine from keratins

The extent of oxidative or hydrolytic decomposition and racemization of cystine (5, 16-19) taking place during protein hydrolysis affects the amount of *l*-cystine available for isolation, while factors interfering with the completeness of isolation of the available compound are: increase of the isoelectric solubility of cystine caused by the presence of inorganic salts (9, 20, 21), of other amino acids (13), humins (10), and other colloidal substances (21), and losses entailed by separation from tyrosine (22) which tends to precipitate together with cystine, as well as by the large amounts

\* A summary of this work appeared in *J Biol Chem*, 105, xcii (1934).

of charcoal (1, 23, 24) usually used for the removal of colored impurities.

Although sulfuric acid as a hydrolyzing agent fell into disrepute for the preparation of cystine evidently because in earlier work cystine was lost with the precipitated  $\text{CaSO}_4$  resulting from lime neutralization of the acid (25), modern work, indicating reduced humin formation (26) and greater stability of cystine (17, 18, 27), makes  $\text{H}_2\text{SO}_4$  appear preferable to  $\text{HCl}$ . We found that when a  $\text{H}_2\text{SO}_4$  hydrolysate is neutralized in such a manner that the solution is saturated with  $\text{Na}_2\text{SO}_4$ , a large part of the brown humin material is salted-out (*cf* (28)), while the precipitate contains practically no cystine. From the filtrate cystine could be removed rapidly, and much more completely than by isoelectric precipitation, as a cupric compound by dilution and neutralization in the presence of  $\text{CuSO}_4$ . The copper precipitate contains, besides cystine (29), chiefly leucine and aspartic acid (30) and a part of the humic acids, while the copper salts of tyrosine and the other amino acids present in wool (31-33) remain in solution (30). The dismutative reaction of Preisler and Preisler (34) does not take place under these conditions. *L*-Cystine hydrochloride may be obtained in good yield by treating the copper precipitate with saturated  $\text{HCl}$  solution, while the inactive modifications tend to stay in solution. Since in preliminary experiments<sup>1</sup> it was found that even the most soluble of the cystine stereoisomers may be completely precipitated by phosphotungstic acid and since of the keratin amino acids forming insoluble cupric salts cystine is the only one (36) precipitable as phosphotungstate from acid solution, isolation of the sum of the optically active and inactive cystine on this basis appeared as a possibility. Up to 80 per cent of the liberated cystine could be isolated in this manner from  $\text{H}_2\text{SO}_4$  hydrolysates, the loss being chiefly due to the difficulty of removing coprecipitated humin phosphotungstates. This contamination increased toward the end of the hydrolysis to such an extent that not more than 31 per cent of the theoretical cystine was obtained by this technique. As external circumstances made it impossible to expand this study into a search for a precipitating

<sup>1</sup> These later developed into a separate investigation the results of which were published in the meantime (35).

agent more selective than phosphotungstic acid, it had to be abandoned at this stage.

#### EXPERIMENTAL

*Precipitation of Cystine from Salt Solution by Cupric Ion*—0.600 gm. of *l*-cystine (2.5 mm) in 5 cc. of 2 *N* HCl and 50 cc. of 3.2 *M* Na<sub>2</sub>SO<sub>4</sub> was precipitated by addition of 10 mm of CuSO<sub>4</sub> in 55 cc. of water and neutralization with NaOH (11.7 cc. of 2.00 *N*) to faintly acid litmus reaction. After standing overnight the precipitate was filtered off and washed free of soluble sulfate. The filtrate showed no optical rotation in a 2 dm. tube, indicating a residual cystine concentration of less than  $5 \times 10^{-4}$  *M*. The copper precipitate (1.547 gm. after drying over P<sub>2</sub>O<sub>5</sub>) gave, dissolved to 50 cc. with *N* HCl,  $[\alpha]_{\text{H}_2\text{O}}^{25}$ , calculated for the total cystine used, =  $-249.9^\circ$ . This figure indicates that more than 98 per cent of the original cystine was present as such in the precipitate, as acid cystine solutions of the same concentration with and without CuSO<sub>4</sub> gave the following values.

5 mm cystine + 10 mm CuSO <sub>4</sub> per 100 cc <i>N</i> HCl,	$[\alpha]_{\text{H}_2\text{O}}^{25} = -249.9^\circ$
5 " " + 20 " " " 100 " " "	$[\alpha]_{\text{H}_2\text{O}}^{25} = -250.0^\circ$
5 " " + (no " ) " 100 " " "	$[\alpha]_{\text{H}_2\text{O}}^{25} = -256.6^\circ$

The composition of the precipitate (cystine 38.8, Cu 36.5, SO<sub>4</sub> 12.5 per cent) indicates the presence of a considerable amount of basic copper sulfate.

*Observations on Action of Strong Sulfuric Acid on Cystine*—For minimizing humin formation in protein hydrolysis, Salkowski (26) advocates the use of 50 per cent (about 14 *N*) sulfuric acid. 1.5 gm. of cystine and 5 cc. of 50 per cent H<sub>2</sub>SO<sub>4</sub> showed after 48 hours heating at 86° a decrease in optical rotation of 31 per cent, indicating a monomolecular racemization constant of the order of 0.8 per cent per hour. An identical solution after 21 hours boiling (126°) showed 6 per cent of the initial rotation, corresponding to a racemization constant of 13 per cent per hour, or approximately a doubling of the reaction rate for every 10°. After 48 hours of boiling the rotation was 0 and the Sullivan test corresponded to 85 to 90 per cent of the initial cystine. According to Salkowski (26) complete hydrolysis may be expected after 2 to 3 hours boiling with 50 per cent H<sub>2</sub>SO<sub>4</sub>, while with constant boiling HCl at least 7

hours are required (14) If for this acid a racemization constant of the order of 4 per cent per hour, calculated from the initial points of the curve of Hoffman and Gortner (16), is assumed, the action of 50 per cent  $\text{H}_2\text{SO}_4$  would seem to compare well with that of constant boiling  $\text{HCl}$ .

*Hydrolysis of Wool with Sulfuric Acid. General*—The wool used throughout was a white degreased commercial grade of 3.21 per cent S, corresponding to a maximum content of 12 per cent cystine. The progress of hydrolysis under various conditions was followed by observations of the optical rotation of the hydrolysate in order to determine whether or not the optical rotation bears any simple relation to the degree of completion of the hydrolysis. Since it was found that decolorizing with charcoal has a considerable effect on the rotation of the hydrolysate (1 00 cc. of a  $\text{H}_2\text{SO}_4$  hydrolysate diluted to 40 cc,  $\alpha = -0.18^\circ$ ; 1 00 cc. of the same hydrolysate diluted to 7 cc, boiled 1 minute with 0.03 gm. of charcoal, washed, and diluted to 40 cc.,  $\alpha = -0.11^\circ$ ), polarimetric readings were taken on the undecolorized hydrolysate after dilution with 39 volumes of water, even though the observational error was high under these conditions. For purposes of comparison the results were expressed as specific rotations of the total wool.

The best response to the biuret reaction was obtained by using 1 cc. of the hydrolysate diluted to twice its volume, adding 5 cc. of concentrated  $\text{NaOH}$ , and stirring in 0.5 cc. of a 3 per cent  $\text{CuSO}_4$  solution. To test the sensitivity of the test under practical conditions 30 gm. of wool + 90 cc. of 50 per cent  $\text{H}_2\text{SO}_4$  were hydrolyzed until the biuret test was negative. By admixture of solutions of peptone or gelatin it was found that with an amount of either of these proteins below 15 per cent of the wool present a positive biuret response can no longer be observed.

In a series of preliminary experiments with Sullivan's test (37) we obtained the best results if the cystine concentration of the unknown solution was within 0.02 and 0.04 gm. per 100 cc. and if this was matched against standards of 0.02 and 0.04 gm. per 100 cc. Under these conditions the average deviation of the readings from their mean value was  $\pm 1.5$  per cent, while the average deviation of the mean value from the theoretical value was  $\pm 4$  per cent. When the unknown concentrations were beyond the range indicated, within 0.01 and 0.08 respectively, and were matched

against 0.02 or 0.04, errors from +30 to -25 per cent were found. The hydrolysates were prepared for the test by diluting with water to an acid concentration of 3 N, boiling for 1 minute with 0.04 gm. of charcoal per 1 cc. of hydrolysate, filtering, washing with 0.1 N HCl, and making up to volume while neutralizing to an acidity of 0.1 N. By repeating the charcoal treatment on the decolorized hydrolysate the correction necessary to discount the loss of cystine in the decolorization was found to be  $+10 \pm 1$  per cent. The same loss caused by charcoal treatment was found on pure cystine solutions.

For colorimetric readings of the cyanide-nitroprusside test 10 cc. of the decolorized and diluted hydrolysate, 5 cc. of 5 per cent NaCN, and after 10 minutes 2.5 cc. of 14 N  $\text{NH}_4\text{OH}$  were combined. The standard was 0.02 gm. of cystine per 100 cc. and the determination was repeated by varying the unknown concentration until standard and unknown fell within 10 per cent of each other. Constant readings were obtained from 15 to 25 minutes after addition of the cyanide. A 10 per cent correction was added to the results.

Hydrolyses with 50 per cent  $\text{H}_2\text{SO}_4$  were carried out with a ratio of 100 gm. of wool to 300 cc. of  $\text{H}_2\text{SO}_4$ . The hydrolysates were worked up by precipitating humins by addition of ice, NaOH, and  $\text{Na}_2\text{SO}_4$  in such amounts that the solution was neutralized to Congo red and saturated with  $\text{Na}_2\text{SO}_4$  at 3 to 5 times the initial volume. After standing overnight the brown humin material floating on the solution was filtered off through a folded filter of high porosity (oil filter) and washed with saturated  $\text{Na}_2\text{SO}_4$  solution. The washed precipitate showed no cyanide-nitroprusside reaction for  $-\text{S}-\text{S}-$ . After addition of excess copper sulfate (about 22 gm. of  $\text{CuSO}_4 + 5\text{H}_2\text{O}$  per 100 cc. of original hydrolysate) the solution was again neutralized at 7 to 8 times the initial volume. The bluish gray copper precipitate was filtered the next day, thoroughly washed by digesting with cold water, and dried on the steam bath. For precipitation of cystine hydrochloride the copper salt was digested with concentrated HCl (about 8 cc. per gm.) and the solution,<sup>2</sup> after complete saturation with HCl gas, was left for

<sup>2</sup> If the solution is too dark, partial removal of colloids by charcoal treatment is necessary lest crystallization of the hydrochloride may be completely inhibited.

several days at room temperature until the crystallization did no longer increase. The hydrochloride was filtered on a fritted glass filter, and washed with alcoholic HCl and ether. From its optical rotation in *N* HCl (38) its content of *l*-cystine hydrochloride was estimated.

Since the attempts to isolate the total cystine of the copper precipitate by phosphotungstic acid made it evident that different commercial preparations of this reagent behave quite differently due to widely varying composition, a detailed account of these experiments is unjustified. The unsatisfactory results obtained led to the separate investigation referred to above on phosphotungstic acid itself and its behavior with the different modifications of cystine (35). In the meantime by empirical treatments of acid solutions of the copper precipitates with phosphotungstic acid, separation of cystine from the precipitated phosphotungstate (39), two reprecipitations by phosphotungstic acid, and final isoelectric precipitation more or less representative fractions of the liberated cystine (95 to 99 per cent pure according to sulfur content) could be isolated (*cf.* Table III)

*Results*—500 gm. of wool and 1500 cc. of 50 per cent  $H_2SO_4$  were heated at  $88^\circ \pm 2^\circ$  for 51 hours, when the biuret test was negative. About 3 hours were required to destroy the wool fiber. The following specific rotations (and average errors) were found.

Hrs	45	65	235	265	30	48	51
$[\alpha]$	-43.3 $\pm 0.4$	-34.4 $\pm 0.4$	-17.3 $\pm 0.8$	-22.0 $\pm 0.8$	-20.4 $\pm 0.7$	-12.6 $\pm 0.7$	-12.6 $\pm 0.7$

The amount of copper salt obtained by working up this hydrolysate in the manner described in the preceding section was 2.0 gm. per gm. of cystine theoretically present in the wool. It yielded (again per gm. of cystine) 0.355 gm. of hydrochloride.  $[\alpha]_{H_2}^{25} = -185^\circ$  (0.500 gm. in 100 cc. of *N* HCl), corresponding to 96.5 per cent of *l*-cystine hydrochloride (38, 40), or a yield of *l*-cystine of 26 per cent of the theory.

That a break in the curve of the optical rotations, as indicated in the preceding experiment, actually occurs was confirmed by observations on a similar hydrolysis (500 gm. of wool + 1500 cc. of 50 per cent  $H_2SO_4$ ,  $81^\circ \pm 3^\circ$ , 45 hours).

Hrs	5	9	13	17	21	24
$[\alpha]$	$-61.8 \pm 1.2$	$-50.4 \pm 1.0$	$-36.1 \pm 0.7$	$-26.8 \pm 0.6$	$-24.7 \pm 0.6$	$-21.6 \pm 0.6$
Hrs	27	30	36	38	42	45
$[\alpha]$	$-17.6 \pm 0.6$	$-17.0 \pm 0.6$	$-20.4 \pm 0.6$	$-20.3 \pm 0.6$	$-19.2 \pm 0.6$	$-18.8 \pm 0.6$

Further experiments showed that under different conditions of hydrolysis similar specific rotation is no indication of a similar extent of amino acid liberation. For instance, 200 gm. of wool were boiled (about  $125^\circ$ ) with 800 cc. of 6 N  $\text{H}_2\text{SO}_4$  for 12 hours, when  $[\alpha]$  was approximately  $-12^\circ$ . The biuret test, however, was distinctly positive, the copper salt obtained was 1.1 gm. per gm. of total cystine, and the hydrochloride formed contained only 6.3 per cent of the total cystine.

Another hydrolysis was conducted at  $50-60^\circ$  for 7 weeks, so that samples could be worked up at various stages in order to see how far the yield of *l*-cystine could be increased. After 150 gm. of wool and 450 cc. of 50 per cent  $\text{H}_2\text{SO}_4$  had been heated for 3 hours on the steam bath ( $75^\circ$ ) the wool was completely immersed and the volume was 550 cc. Protected by a condenser and  $\text{CaCl}_2$  tube the solution was now kept at  $50-60^\circ$ . The first sample (21 days) was taken when the biuret reaction had become definitely negative. Table I shows the time when samples (150 cc. each) were removed, the specific rotation, the amounts of copper salt and hydrochloride obtained per gm. of total cystine corresponding to the sample, the purity of the hydrochloride in terms of *l*-cystine hydrochloride, according to optical rotation, and the *l*-cystine obtained as hydrochloride in terms of total cystine. This experiment shows that even after the biuret test has become negative the amount of *l*-cystine available continues to increase.

In the next hydrolysis, instead of precipitating the *l*-hydrochloride, an attempt was made to obtain the total cystine contained in the copper salt by means of phosphotungstic acid. 600 gm. of wool were heated with 1800 cc. of 50 per cent  $\text{H}_2\text{SO}_4$  in an oil bath without stirrer on a hot-plate. The temperature was regulated by a de Khotinsky thermostat and, measured within the flask, varied between  $75-83^\circ$ . Readings, taken twice a day, averaged  $79^\circ \pm 2^\circ$  for the first 10 days,  $77^\circ \pm 2^\circ$  for the second 10



days, and  $81^{\circ} \pm 2^{\circ}$  for the remainder. Table II shows yield and analytical results of copper salts isolated at various stages in the manner previously described. Free sulfate and total sulfur (by a modification of the method of Blix (41)) were determined after precipitation of the copper with  $H_2S$  and removal of  $H_2S$  by  $CO_2$ . A comparison of the results of Table II with those obtained on a

TABLE I  
*Isolation of Copper Salts and l-Cystine Hydrochloride from Hydrolysate*

Sample No	Time	$[\alpha]$	Cu salt per gm of cystine	Hydrochloride per gm of cystine	l-Cystine hydrochloride in total hydrochloride	l-Cystine obtained
	days	degrees	gm	gm.	per cent	per cent
1	21	-19.2	1.63	0.424	95	30.9
2	43	-6.0	2.15	0.615	87	41.0
3	49	-9.6	2.22	0.588	97	43.7

TABLE II  
*Analysis of Copper Precipitates from Hydrolysate*

Sample No	Time	Amount per gm of total cystine of wool	Cu content	Total S content	$SO_4$ content	Organic S content		
						S	S calculated as cystine	
							Fraction of Cu salt	Fraction of total cystine of wool
	days	gm	per cent	per cent	per cent	per cent	per cent	per cent
1	3	1.59	19.2	11.0	0.66	10.8	40.5	66
2	7	2.27	19.2	10.1	0.78	9.8	36.5	86
3	12	2.18	19.0	10.1	0.69	9.9	37.0	83
4	16	2.21	18.9	9.9	0.66	9.7	36.5	82
5	33	2.53	18.6	9.6	0.33	9.5	35.5	92

sodium sulfate solution of pure cystine (p. 41) shows that the organic sulfur content (cystine) is similar in both cases, while the precipitate from the hydrolysate contains almost no sulfate and only about one-half as much copper as the precipitate from the pure solution. Also only 20 to 25 per cent of the copper used reappeared in the precipitate as compared with 89 per cent in the former case. Apparently soluble copper salts of other amino acids

play a rôle in the hydrolysate in preventing precipitation of basic copper sulfates, which is considerable in the pure solution. On the other hand the copper present in excess over the atomic ratio of copper to organic sulfur = 1:2 (the ratio of a cupric cystinate), while attributable to basic sulfate in the pure solution, may be accounted for in the hydrolysate by the coprecipitation of the insoluble (30) copper salts of leucine and aspartic acid.

The results of Sullivan determinations on the hydrolysate are plotted in Fig. 1. Comparison of the percentages of free cystine

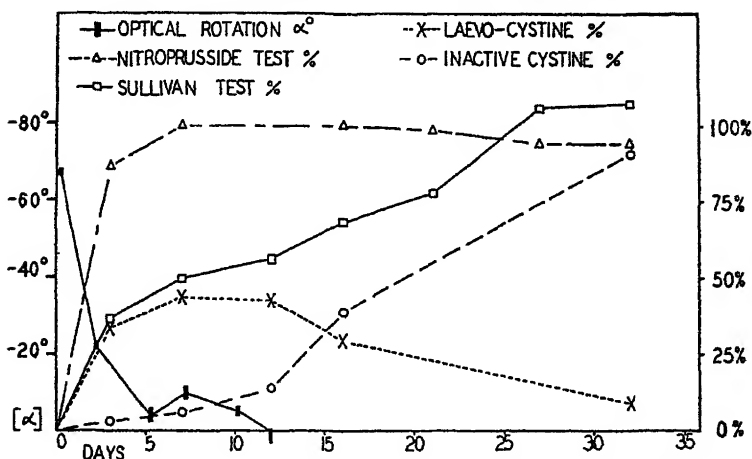


FIG 1 Hydrolysis of wool with 50 per cent sulfuric acid at 80°. The figures for optical rotation, nitroprusside, and Sullivan test were obtained in the manner described under "Hydrolysis of wool with sulfuric acid, General". The data underlying the curves for *l*- and inactive cystine are contained in Table III.

thus obtained with the cystine percentages calculated from the organic sulfur of the copper precipitates (Table II) on the one hand, and on the other hand, with the reactive disulfide obtained by the nitroprusside method (also plotted in Fig. 1) indicates that part of the cystine in the copper precipitates must be present in the form of cystine peptides in which the disulfide group is reactive while they do not respond to the free cystine reaction of Sullivan.

Table III summarizes the results of cystine isolations from the copper salts of Table II by means of phosphotungstic acid. The

calculated values in Columns 5 and 6 were obtained on the two limiting assumptions, either (upper limit) that the *l*-cystine content of the unisolated fraction of the liberated cystine is the same as that of the isolated fraction (Column 4), or (lower limit) that the unisolated portion is all inactive cystine. However, in Sample 4, where two isolations with resulting different yields were carried out, it is clear that the true value must be between 33 and 27 per cent; *i.e.*, in case of higher yield the correct value is near the maximum and in case of lower yield near the minimum. On this basis the values of Column 7 were estimated, which are nothing more than best approximations. They are plotted in the *l*-cystine

TABLE III  
*Isolation of Cystine from Copper Salts by Phosphotungstic Acid*

Sample No	Cystine isolated from Cu salts by phosphotungstic acid			Free <i>l</i> -cystine in solution (based on total S in wool)		
	Yield based on total S in wool	Yield based on amount liberated according to Sullivan test	<i>l</i> -Cystine content according to specific rotation	Upper calculated limit	Lower calculated limit	Estimated value
(1)	(2)	(3)	(4)	(5)	(6)	(7)
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	28	75	97	36	27	34
2	40	81	93	46	38	44
3	40	71	82	47	33	43
4	35	51	77	(53)	27	30
	49	74	48	33	(25)	
5	31	31	27	27	8	9

curve of Fig. 1. The curve for inactive cystine in Fig. 1 represents the difference between the total liberated cystine and the estimated free *l*-cystine.

In this hydrolysis the biuret test was slightly positive on the 5th day but negative on the 7th day. The control experiments mentioned above (p. 42) showed that the sensitivity of the biuret reaction is greatly decreased by the dark color of the hydrolysate. Decolorization, on the other hand, would involve danger of losing protein material, and it seems evident, therefore, that a negative biuret test may be quite misleading as a criterion for the completeness of wool hydrolysis, for which it seems to be used frequently

even though the unreliability of the test was already pointed out by Osborne and Jones in 1910 (42). The discrepancy of about 13 per cent between the nitroprusside and the Sullivan test, confirmed by duplicate determinations, in the final stage, where they should coincide at 100 per cent, is probably due to errors inherent in the methods. Owing to racemization paralleling the liberation of cystine the amount of *l*-cystine, as was to be expected, goes through a maximum. In the beginning cystine seems to be liberated entirely in the levorotatory form. The apparent increase in the rate of formation of inactive cystine suggests that racemization may take place within the peptide linkage preceding complete liberation, but with the present degree of accuracy no definite conclusion can be drawn. The significance of the observed break in the optical curve cannot be appraised without further work. However, it seems to be a phenomenon beyond the limits of observational error, especially since curves with a similar characteristic turn were found on hydrolyses under different conditions.

#### SUMMARY

In the preparation of cystine by hydrolysis of wool 50 per cent sulfuric acid was found to be a suitable hydrolyzing agent. A large humin fraction can be removed from the hydrolysate by salting-out with  $\text{Na}_2\text{SO}_4$  and cystine can be rapidly, and without oxidative or reductive changes, precipitated by cupric ion. The best yield of *l*-cystine, obtained by converting the cystine contained in the copper precipitate into the hydrochloride, was equivalent to 44 per cent of the total sulfur content of the wool. A negative biuret test or a cyanide-nitroprusside test corresponding to the total sulfur content of the wool is no proof of completeness of hydrolysis as far as liberation of cystine is concerned. Due to racemization, however, the stage at which the amount of *l*-cystine available for isolation is at its maximum does not coincide with the completion of hydrolysis. Under different conditions of hydrolysis no relation was found between the optical rotation of the hydrolysate and the state of cystine.

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## SPECTROPHOTOMETRIC STUDIES

### II. PREPARATIONS FROM WASHED BLOOD CELLS; NITRIC OXIDE HEMOGLOBIN AND SULFHEMOGLOBIN

BY DAVID L. DRABKIN AND J. HAROLD AUSTIN

*(From the Department of Physiological Chemistry and the John Herr Musser  
Department of Research Medicine, School of Medicine, University of  
Pennsylvania, Philadelphia)*

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In this paper evidence will be presented which indicates that spectrophotometric constants are more precisely reproducible with solutions prepared from washed erythrocytes than from hemolyzed whole blood, used in our earlier analyses (1). The data, obtained under standard conditions which will be defined, include the absorption curves of  $\text{HbO}_2$ , Hb,  $\text{HbCO}$ ,  $\text{MHbCN}$ ,  $\text{HbNO}$  (nitric oxide hemoglobin), and  $\text{SHb}$  (sulfhemoglobin). The two latter pigments were the main subjects of the investigation.

$\text{HbNO}$  was prepared under conditions which excluded the presence of higher oxides of nitrogen and the possible formation of  $\text{MHb}$ . These precautions were not taken in previous studies of  $\text{HbNO}$  (2) and rendered doubtful the older spectroscopic data.

$\text{SHb}$  probably has not been prepared in a pure state, as claimed (3). We have derived the spectrum of this pigment by extrapolation from absorption data upon mixtures of Hb and  $\text{SHb}$ . We were thus able to establish the identity of the abnormal pigment present in the blood of a patient with clinical sulfhemoglobinemia (4).

#### *Methods*

The spectrophotometer and technique employed were those described in our previous publication (1), except for the following modifications.

*Preparation of Hemoglobin Solutions*—Dog blood was obtained

by cardiac puncture from normal, unfed animals. It was defibrinated. The serum was separated by centrifuging and the cells were washed three times with about 4 volumes of 0.9 per cent saline. The washed erythrocytes were hemolyzed by the addition of enough distilled water to make 10 times the original volume of blood employed. This solution was filtered to remove cell debris. It was kept on ice and was not used longer than for a period of 4 days. From the 1:10 stock solution the more dilute solutions (1:50 or 1:100) were prepared.

*Notation*—In our absorption curves  $\epsilon$  values are plotted both against wave-length,  $\lambda$ , in  $m\mu$ , and wave number,  $\nu$ , which may be defined as the *frequency per cm.* (5) and which is the reciprocal of  $\lambda$  expressed in centimeters ( $\nu = 1/(\lambda \text{ in } m\mu \times 10^{-7})$ ). Although most spectrometers are calibrated in terms of  $\lambda$ ,  $\nu$  is more useful in the interpretation of spectroscopic data (6).

Our extinction coefficients,  $\epsilon$ , are qualified by the concentration of pigment,  $c$ , expressed in mm per liter where 1 M indicates 1 mole of porphyrin, which for hemoglobin is equivalent to 1 gm. atomic weight of Fe or 1 mole of  $O_2$  capacity. Standard values are given as  $\epsilon(c = 1 \text{ mm per liter})$ , obtained from the equation

$$\epsilon(c = 1 \text{ mm per liter}) = \frac{\epsilon_{\text{observed}}}{d(\text{depth})_{\text{cm}} \times c(\text{actual concentration in mm per liter})}$$

$\epsilon$  values at standard concentrations are far more useful than the archaic absorption constant  $A = c/\epsilon$ . The use of molar units for  $c$  permits ready comparison of the absorption spectra of all derivatives and relatives of hemoglobin.

For comparison with Table III of our earlier paper (1)  $\epsilon(c = 1 \text{ mm per liter}) = 1/60A = 167/(A \times 10^4)$ . In Fig. 1 of that paper the ordinates designated as  $1/A$  are actually  $1/(A \times 10^4)$ . For convenience we have introduced at the right of our graphs a scale of  $\epsilon(c = 1 \text{ gm. Hb per 100 cc}) = \frac{1}{1.67} \epsilon(c = 1 \text{ mm per liter})$ .

*Quantitative Estimation of Pigment Concentration*—Total pigment was determined by measuring the absorption of cyanmethemoglobin (cyanhemoglobin), MHbCN, prepared from suitable aliquots by the addition of  $K_3Fe(CN)_6$  to a final concentration of 0.6 mm per liter and KCN to a concentration of 0.8 mm per liter, the pigment concentration being of the order of 0.1 to 0.2 mm per

liter. The constants for MHbCN which were utilized in this determination are:  $\epsilon(c = 1 \text{ mm per liter}) = 11.1$  at  $\lambda 551$ ,  $11.5$  at  $\lambda 545$ , and  $11.5$  at  $\lambda 540 m\mu$  (1). At these wave-lengths the molar extinction coefficient of  $K_3Fe(CN)_6$  has an average value of only  $0.28$  ( $\epsilon(c = 1 \text{ mm per liter}) = 0.00028$ ); the absorption of  $K_4Fe(CN)_6$  in this spectral region is even less. Hence no correction was made for the absorption of ferri- or ferrocyanide. The use of the absorption of MHbCN as a standard of reference has the following advantages. We have attained a higher precision of measurement of hemoglobin spectrophotometrically (1) than by gasometric technique. It is the most direct procedure, and avoids such highly questionable practices as the estimation of total pigment colorimetrically as a basis for absorption constants (7). MHbCN is an ideal pigment for the determination of total concentration. Hb, HbO<sub>2</sub>, HbCO, HbNO (Fig. 2), and MHb are all readily convertible into MHbCN. Within fairly wide limits (Fig. 1) pH does not influence the conversion or the spectrum, and the characteristic absorption of MHbCN is maximal in the region of greatest visual sensitivity.

Very recently Brooks (8) published her conclusion "that the absorption curve for cyanhemoglobin was identical with that for oxyhemoglobin." She apparently studied mixtures rather than single pigment derivatives and drew her conclusion from a consideration of measurements at only two wave-lengths, calculating the ratio of extinction coefficients at  $\lambda 560$  and  $\lambda 540 m\mu$ . The use of extinction ratios at two points in the spectrum has been advocated as a guide to the detection of impurities in HbO<sub>2</sub> and not for the identification of pigments. Consideration of the shape of the entire spectrophotometric curves of HbO<sub>2</sub> and MHbCN (Fig. 1), in fact so different in their character, would, we believe, have led her to a different conclusion.

*Preparation of HbNO and SHb*—In the preparation of HbNO the absence of oxygen (to avoid the formation of higher oxides of N<sub>2</sub>) was insured by the use of reduced hemoglobin solutions and exposure to NO in an atmosphere of nitrogen. Washed defibrinated dog erythrocytes were hemolyzed in water to 10 times the volume of the initial blood and filtered. 20 cc. of this solution + 20 cc. of 133 mm per liter of phosphate buffer (pH 7.0) were diluted to 100 cc. The diluted solution was repeatedly evacuated in a



tonometer (capacity of 400 cc.) and equilibrated with  $\text{CO}_2 + \text{N}_2$ , then with  $\text{N}_2$ . The solution was filtered and again repeatedly evacuated and equilibrated. A sample was converted to MHbCN for determination of concentration, another sample was run into the spectroscopic cell with minimal exposure to air and read to establish completeness of reduction. Reduction having been demonstrated, successive additions of NO were run into the tonometer, the diluting gas being  $\text{N}_2$ . As equilibrations with successive increases in NO tension were completed, samples were quickly sealed in the spectroscopic cell for reading. The NO was generated by delivering 33 per cent  $\text{HNO}_3$  upon thin strips of pure Cu suspended in an air-tight cylinder over water. The cylinder and connection were previously evacuated and flushed with  $\text{N}_2$ . Details of special experiments appear in the legends accompanying the figures.

SHb was prepared with  $\text{H}_2\text{S}$  generated in a Kipp apparatus with HCl and washed in water. The gas was delivered at a rate of approximately 150 bubbles per minute from an outlet tube of 3 mm. bore. The foaming of the dilute solutions of hemolyzed washed blood cells receiving the gas was minimized with minute amounts of caprylic alcohol. The duration of exposure to  $\text{H}_2\text{S}$  was variable and is indicated in the legends accompanying the figures. Alternate exposure to  $\text{H}_2\text{S}$  and air or oxygen (3) was found unnecessary, sufficient oxygen being furnished by carrying out the reaction in open flasks.

The pH of solutions was determined by means of the glass electrode.

### *Results*

Fig 1 shows the absorption curves of solutions of  $\text{HbO}_2$ , HbCO, Hb, and MHbCN, prepared from hemolyzed, washed erythrocytes. It is evident that with these pigments fairly wide variations in pH did not affect the absorption curve. Table I indicates that greater precision of measurement was attained upon solutions prepared from hemolyzed, washed blood cells than from hemolyzed whole blood. The ratios of absorption of the peaks of the curve to the trough were somewhat higher for  $\text{HbO}_2$  and HbCO prepared by the present technique than for the corresponding pigments prepared from whole blood (Table I, last two columns).

The differences in the two sets of preparations are most probably related to physical factors such as very slight turbidity, unavoid-

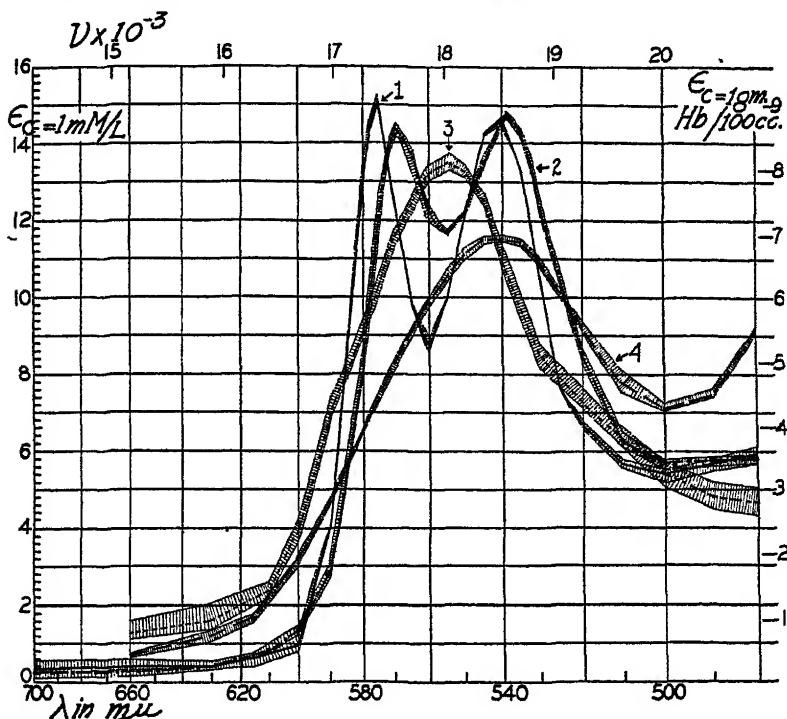


FIG. 1. Absorption curves of  $\text{HbO}_2$ ,  $\text{HbCO}$ ,  $\text{Hb}$ , and  $\text{MHbCN}$ , cross-hatched to show the spread of the determinations. Curve 1 represents  $\text{HbO}_2$  (eight solutions) 0.070 to 0.205 mm per liter, buffers 33 mm per liter of phosphate, 25 mm per liter of borate (two unbuffered solutions), pH 5.9 to 9.2, Curve 2,  $\text{HbCO}$  (six solutions) 0.0942 to 0.205 mm per liter, buffers 33 mm per liter of phosphate, 10 to 27 mm per liter of borate (one unbuffered solution and one with 100 mm per liter of  $\text{NH}_4\text{OH}$ ), pH 5.9 to 11.0; Curve 3,  $\text{Hb}$  (seven solutions) 0.107 to 0.205 mm per liter, buffers 13 to 27 mm per liter of phosphate, 24 mm per liter of borate (two unbuffered solutions), pH 4.5 to 9.2. Reduction was by evacuation in one solution, in the others with  $\text{Na}_2\text{S}_2\text{O}_4$ , 4 mm per liter; Curve 4,  $\text{MHbCN}$  (seven solutions) 0.113 to 0.205 mm per liter, buffers 15 to 33 mm per liter of phosphate, 11 to 25 mm per liter of borate (one unbuffered solution), pH 5.9 to 9.2.  $\text{K}_3\text{Fe}(\text{CN})_6$  0.8 to 0.9 mm per liter and  $\text{KCN}$  0.7 to 0.8 mm per liter.

able when whole blood is used. It is well recognized that lower ratios for  $\text{HbO}_2$  will be obtained when  $\text{MHb}$  is present, but it is

## 56 . Spectrophotometry of HbNO and SHb

TABLE I  
Precision of Measurement of Absorption Constants

Pigment	Source*	No. of specimens	$\epsilon(c = 1 \text{ mm per liter})$						$\sqrt{\frac{2s^2}{n-1}}$	Ratio, $\epsilon/\epsilon_{\dagger}$	
			$\lambda$	Average	High	Low	$\Delta$ , high to low	$\lambda \frac{575 \text{ m}\mu}{\lambda \frac{560 \text{ m}\mu}$		$\lambda \frac{540 \text{ m}\mu}{\lambda \frac{560 \text{ m}\mu}$	
HbO <sub>2</sub>	A	17	$\text{m}\mu$ 575	15 79	16 7	15 3	1 4	0 31	$\pm$ 0 05	1 67	1 62
"	B	8	560	9 43	10 2	9 0	1 2	0 26	$\pm$ 0 04		
			540	15 29	16 5	14 8	1 7	0 38	$\pm$ 0 07		
			575	15 13	15 26	15 06	0 20	0 07	$\pm$ 0 02	1 73	1 68
HbCO	A	16	560	8 73	8 95	8 56	0 39	0 13	$\pm$ 0 03		
			540	14 62	14 70	14 52	0 18	0 06	$\pm$ 0 01		
			569	14 71	15 48	13 22	2 26	0 48	$\pm$ 0 08	1 22	1 23
"	B	6	558	12 09	12 50	11 80	0 70	0 29	$\pm$ 0 05		
			539	14 83	15 64	13 31	2 33	0 49	$\pm$ 0 09		
			569	14 39	14 54	14 08	0 46	0 16	$\pm$ 0 05	1 23	1 26
Hb	"	7	558	11 81	11 92	11 70	0 22	0 08	$\pm$ 0 02		
			555§	11 70	11 74	11 65	0 09	0 05	$\pm$ 0 02		
			539	14 77	14 82	14 69	0 13	0 05	$\pm$ 0 01		
MHbCN	"	7	560	13 24	13 51	13 00	0 51	0 21	$\pm$ 0 06		
			555	13 50	13 66	13 28	0 38	0 14	$\pm$ 0 04		
			550	13 28	13 45	13 12	0 33	0 12	$\pm$ 0 03		
		7	551	11 10	11 28	10 97	0 31	0 10	$\pm$ 0 03		
			545	11 52	11 60	11 40	0 20	0 08	$\pm$ 0 02		
			540	11 53	11 63	11 47	0 16	0 05	$\pm$ 0 01		

\* Solutions prepared from hemolyzed, whole dog blood are listed as Source A, those prepared from hemolyzed, washed dog erythrocytes, as Source B.

† The figures after the  $\pm$  represent the standard error of the standard deviation.

‡ Ratios of extinction coefficients at the maxima of absorption to the minimum between the two bands.

§ Only three determinations at this wave-length were made.

|| The average constants have been assumed on the basis of our earlier work (1) so that only the spread of determinations is of significance in the case of this pigment

not justifiable to conclude (9) that admixture with the oxidized form of hemoglobin is the sole cause of this phenomenon. The reasons for this will be made more apparent in two of the following papers.

Fig. 2 is typical of the data obtained in experiments with HbNO. The absorption curve of HbNO is evidently that of an analogue of HbO<sub>2</sub>. The spectrum is distinguishable from that of HbO<sub>2</sub> by the shallowness of the trough between the two max-

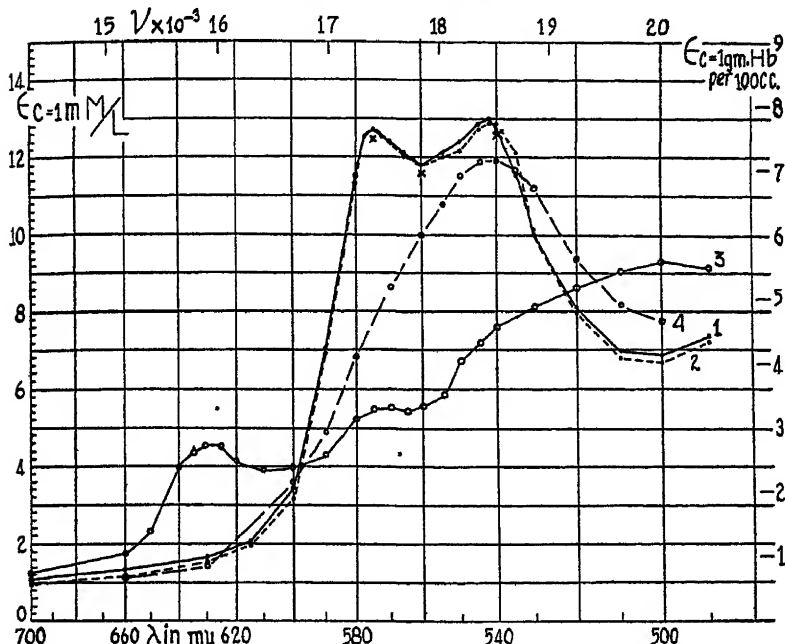


FIG 2 HbNO and its conversion to MHb and MHbCN. Curve 1 represents HbNO, 0.204 mm per liter, PO<sub>4</sub> buffer (pH 7.0) 25 mm per liter, pH 7.1, tension of NO 87 mm. of Hg, Curve 2, HbNO, 0.204 mm per liter, PO<sub>4</sub> buffer (pH 7.0) 25 mm per liter, pH 7.0, tension of NO 244 mm. of Hg; Curve 3, MHb, 0.186 mm per liter, prepared from HbNO solution with K<sub>3</sub>Fe(CN)<sub>6</sub>, 3.6 mm per liter; Curve 4, MHbCN, 0.168 mm per liter, prepared from the above MHb solution with KCN, 1.4 mm per liter. The cross marks are for HbNO, the solution of Curve 2 after evacuation and equilibration with CO<sub>2</sub> + N<sub>2</sub>.

ima of absorption. This results in unusually low ratios of extinction coefficients of peaks to trough.

$$\frac{\epsilon_{\lambda 575 \text{ m}\mu}}{\epsilon_{\lambda 560 \text{ m}\mu}} = 1.08$$

$$\frac{\epsilon_{\lambda 542 \text{ m}\mu}}{\epsilon_{\lambda 560 \text{ m}\mu}} = 1.10$$

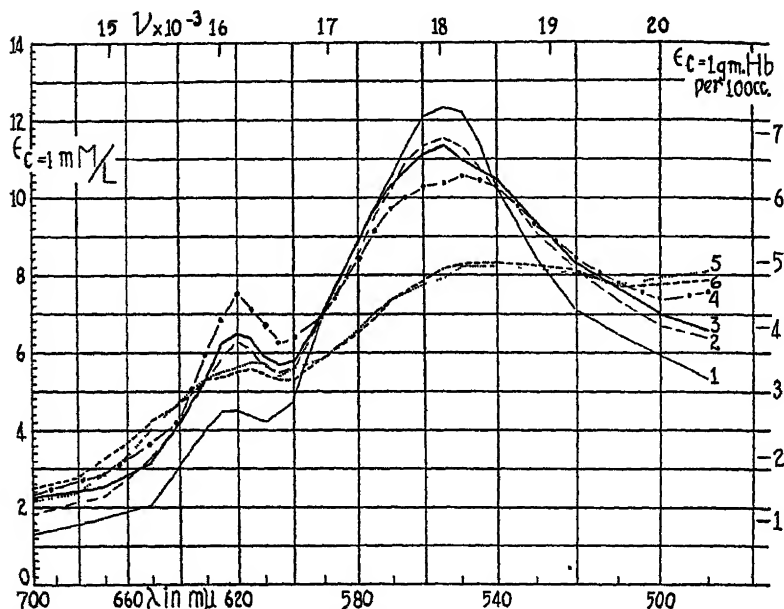


FIG 3. Effect of duration of treatment with  $H_2S$  upon absorption curve of hemoglobin solutions. Curve 1 represents 4 minutes exposure to  $H_2S$ , interpreted as 69 to 74 per cent Hb and 31 to 26 per cent SHb. Total pigment 0.201 mm per liter, borate buffer (pH 9.2) 10 mm per liter. Curve 2, 8 minutes exposure to  $H_2S$ , interpreted as 47 to 55 per cent Hb and 53 to 45 per cent SHb; Curve 3, 8 minutes exposure to  $H_2S$  followed by evacuation, interpreted as 44 to 52 per cent Hb and 56 to 48 per cent SHb. No change on adding  $Na_2S_2O_4$  or KCN; Curve 4, 50 minutes exposure to  $H_2S$ , interpreted as 28 to 37 per cent Hb and 72 to 63 per cent SHb. Total pigment 0.158 mm per liter, borate buffer (pH 9.2) 10 mm per liter; Curve 5, 90 hours exposure to  $H_2S$ . Total pigment at start 0.171 mm per liter, phosphate buffer (pH 7.0) 13.3 mm per liter, final pH 6.91. Centrifuged and filtered to remove copious precipitate, mainly S; Curve 6, 4 hours exposure to mixed  $H_2S$  and air and 86 hours exposure to  $H_2S$ . Total pigment at start 0.171 mm per liter, phosphate buffer (pH 7.0) 13.3 mm per liter, final pH 6.83. Centrifuged and filtered to remove precipitate.  $\epsilon$  values raised by 13.8 per cent to bring level of curve to that of preceding solution, assuming greater loss of pigment.

The locations of the absorption maxima are not materially different than for  $HbO_2$ . The apparent slight shift of the  $\beta$  band towards the red may be significant, but our data are insufficient to establish this point.

HbNO was quantitatively converted by the addition of  $K_3Fe(CN)_6$  into MHb. In the experiment reported in Fig. 2 the conversion was at pH 7.0 and the spectrum obtained is very similar to that yielded by MHb prepared from  $HbO_2$  at the same H ion concentration (10). The quantitative conversion of the MHb formed from HbNO into MHbCN was also demonstrated. This would appear definitely to establish HbNO as a true analogue of  $HbO_2$ .

HbNO, buffered at pH 7.0, was not converted to Hb (Fig. 2) by repeated evacuation and equilibration with  $CO_2 + N_2$ , then with  $N_2$ —conditions under which  $HbO_2$  would have been completely reduced. The union of Hb with NO is apparently more firm than with  $O_2$ . HbNO appeared also to be more stable to alteration in H ion concentration than  $HbO_2$ . We observed no changes in spectrum in the wide pH range of 3.4 to 11.2.

Fig. 3 presents the data obtained in a group of experiments in which  $HbO_2$  was exposed to  $H_2S$  for varying periods of time. In Curves 1 to 4 it is obvious that there is a progressive change in spectrum involving simultaneously an increase of absorption in the red at  $\lambda$  620  $m\mu$  and a decrease in the green at  $\lambda$  555  $m\mu$ . A consideration of the absorption curve of Hb (Fig. 1) led to the assumption that at least Curves 1 to 3 of Fig. 3 were due to mixtures of Hb and SHb. It was assumed at first that Curve 4 (50 minutes exposure to  $H_2S$ ) represented complete conversion to SHb.

Curves 5 and 6 (Fig. 3) were obtained after long exposure of hemoglobin to  $H_2S$ , followed by centrifuging and filtration to remove copious precipitates mainly of colloidal S. Precipitation with the possible loss of pigment invariably occurred when solutions of  $HbO_2$  were exposed to  $H_2S$  for 1 hour or longer. The precipitate from the solution represented by Curve 5 had no appreciable color. There was a greater loss of pigment from the solution represented by Curve 6 (exposed actively to  $H_2S$  and air), and the level of this curve has been adjusted to the concentration of the pigment in the solution represented by Curve 5. Both solutions gave optical evidence (Tyndall effect) of slight turbidity. The shape of these absorption curves sets them apart from the others reported in Fig. 3.<sup>1</sup> The proportional decrease

<sup>1</sup> The exact level of the curves is uncertain, since the concentration of pigment in these experiments could not be determined by conversion to

in the two maxima of absorption in comparison with Curve 4 and a relative increase in absorption at the red and blue ends of the spectrum could be due to a combination of the factors of loss of pigment and presence of turbidity. This explanation, however, demands a greater loss of pigment than appears to have actually taken place and does not account for the shift of the absorption maxima towards the blue. It is, therefore, also probable that, after long exposure to  $\text{H}_2\text{S}$ , secondary pigment changes may have occurred. We believe that Haurowitz's SHb studies (3) may be interpreted in a similar manner. Our curves are not inconsistent with the changes in spectrum which would occur were appreciable amounts of a hemin-like derivative (11) produced.

Examples of the absorption spectra yielded by the hemolyzed blood of a patient with clinical sulfhemoglobinemia<sup>2</sup> as well as of specially prepared control mixtures of normal  $\text{HbO}_2$  treated with a solution of  $\text{H}_2\text{S}$  are presented in Fig. 4 (Curves 1 and 2). It is evident that these curves are similar. However, when we constructed theoretical curves of mixtures of  $\text{HbO}_2$  and SHb, assuming that Curve 4 of Fig. 3 (repeated in Fig 4 for convenience) was pure SHb, we failed to get any curve the shape of which resembled the curves yielded by the pathological blood and the control blood treated with  $\text{H}_2\text{S}$  water. This led us to the conclusion that Curve 4, like Curves 1 to 3 in Fig. 3, was that of a mixture of Hb and SHb. This curve represented the maximum conversion to SHb attained in our experiments before precipitation and perhaps other changes occurred.

The absorption spectrum of SHb (Curve 4, Fig. 4) has been deduced mathematically from the data at four wave-lengths,  $\lambda$  620,  $\lambda$  575,  $\lambda$  560, and  $\lambda$  540  $m\mu$ , for Curves 1 to 4 in Fig. 3. The underlying assumption was that these curves represented a

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MHbCN. The addition of KCN alone produced no change in spectrum, while the addition of  $\text{K}_2\text{Fe}(\text{CN})_6$  resulted in the precipitation of highly pigmented material.

<sup>2</sup> The patient had chronic acetanilide poisoning, in this respect resembling one of the patients described by Harrop (4). Solutions prepared from the blood showed spectroscopically the usual bands of  $\text{HbO}_2$  and besides a band at  $\lambda$  620  $m\mu$ . Most of the pigment in the blood was convertible into Hb by means of  $\text{Na}_2\text{S}_2\text{O}_4$  and into MHbCN by the addition of  $\text{K}_2\text{Fe}(\text{CN})_6$  and KCN, but the band in the red persisted unaltered in strength in each case.

progressive change from Hb to SHb. We determined by successive approximations the values for the concentration of Hb ( $c_{\text{Hb}}$ ) and SHb ( $1 - c_{\text{Hb}}$ ) for each of the four solutions and the

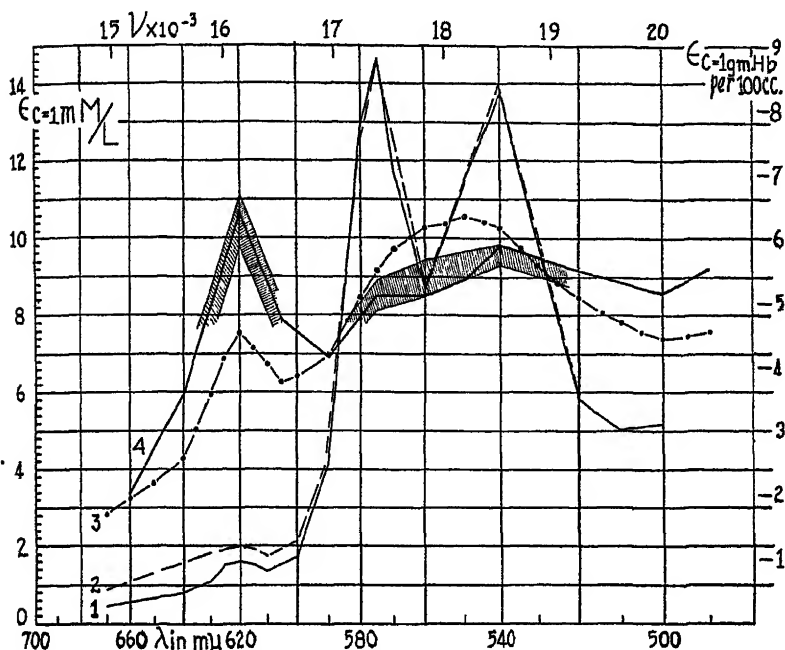


FIG 4 Absorption spectrum of blood of a patient with clinical sulfhemoglobinemia and our extrapolated spectrum of pure SHb. Curve 1 represents pathological blood. Total pigment 0.141 mm per liter, HbO<sub>2</sub> 0.124 to 0.126 mm per liter, SHb 0.015 to 0.017 mm per liter; Curve 2, control from normal human blood treated with H<sub>2</sub>S. Total pigment 0.226 mm per liter, as determined both upon original HbO<sub>2</sub> and after conversion, HbO<sub>2</sub> 0.192 to 0.196 mm per liter, SHb 0.030 to 0.035 mm per liter. Prepared by adding 0.1 cc of a saturated aqueous solution of H<sub>2</sub>S to 5 cc of dilute solution of hemolyzed washed erythrocytes; Curve 3, Hb + SHb, same as Curve 4, Fig 3; Curve 4, extrapolated spectrum of SHb (see text). Cross-hatched areas indicate zone of uncertainty

absorption constants of SHb ( $\epsilon_{\text{SHb}}$ ) at the four chosen wavelengths which satisfied best sixteen equations which can be set up under these circumstances. The equations were of the form

$$c_{\text{Hb}} \times \epsilon_{\text{Hb}} + (1 - c_{\text{Hb}}) \times \epsilon_{\text{SHb}} - \epsilon_{\text{observed}} = 0$$



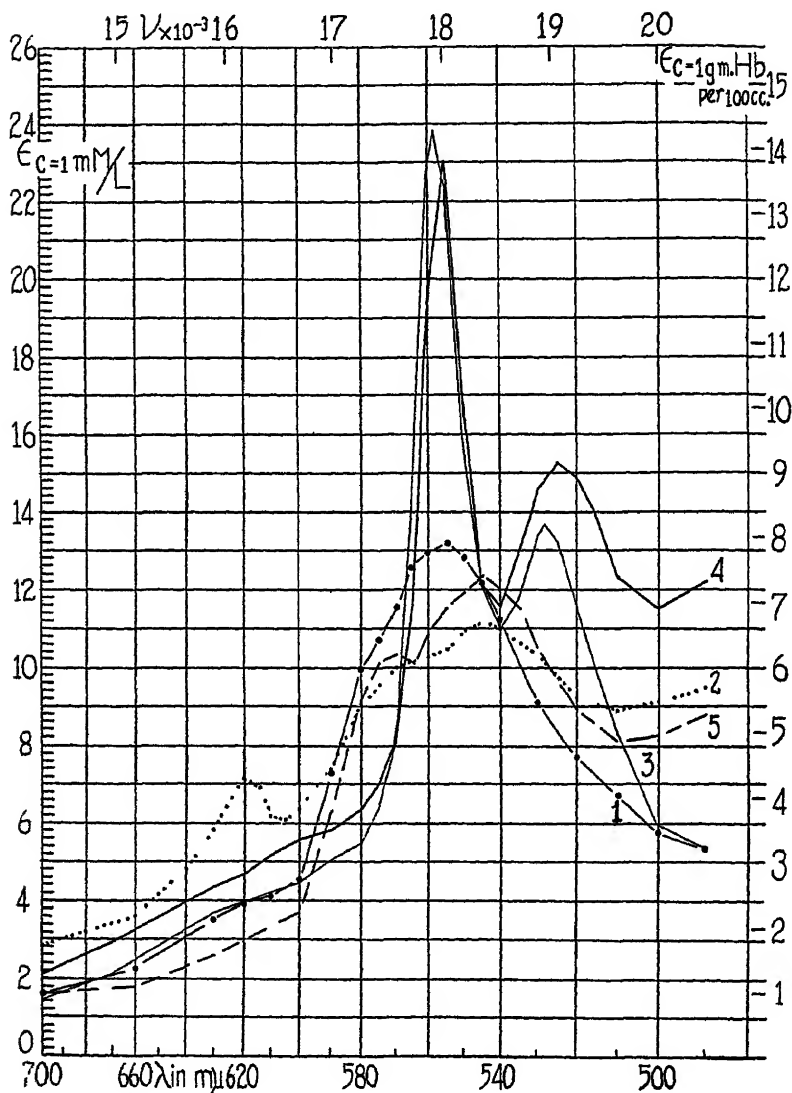


FIG 5 Curves for reduced hemochromogens, prepared from mixtures of Hb and SHb, and for sulfmethemoglobin, SMHb. Curve 1 represents Hb exposed to  $H_2S$ . Total pigment 0.151 mm per liter, phosphate buffer (pH 7.0) 13.3 mm per liter, reduced in tonometer by evacuation and equilibration

The sixteen equations contained eight unknowns. The data permitted each of the unknowns to be evaluated within certain limits. The  $\epsilon_{\text{SHb}}$  values so obtained were used to plot our deduced spectrum of pure SHb, the cross-hatched areas indicating the limits of the approximation. Proof of the validity of our assumptions was furnished by the ability to construct theoretical curves of mixtures of HbO<sub>2</sub> and SHb or of Hb and SHb which agreed excellently with the observed spectra from the patient's blood, both before and after the addition of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, and with normal hemolyzed blood treated with a solution of H<sub>2</sub>S. The percentage composition of the mixtures of pigments which appear in the legends of Fig 3 and 4 and the total pigment concentration of the clinical case were calculated from the absorption data of HbO<sub>2</sub> and Hb (Fig 1) and of SHb (Fig. 4). This is, we believe, the first time that quantitative estimation of sulfhemoglobin concentration in a clinical case has been accomplished.

No consistent changes in the absorption spectra of mixtures of Hb and SHb took place with variations in pH from 6.2 to 10.4. It is of interest, however, that solutions of hemoglobin through which H<sub>2</sub>S was bubbled became somewhat more acid. The acidity of H<sub>2</sub>S ( $K_1 = 9.0 \times 10^{-10}$ ) may partly account for a marked drop in pH, 9.2 to 6.7, observed in solutions buffered with Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>. This explanation, however, cannot be applied to a slight though consistent lowering of pH, 7.0 to 6.83 and 7.0 to 6.23, of solutions buffered with phosphate. The possibility that acid was produced by the oxidation of H<sub>2</sub>S must be considered.

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with CO<sub>2</sub> + N<sub>2</sub>, then with N<sub>2</sub>, equilibrated with pure H<sub>2</sub>S in tonometer, transferred to cell with minimal exposure to air, and read, Curve 2, Hb + SHb, preceding solution after exposure to air; Curve 3, reduced hemochromogen. Total pigment 0.087 mm per liter, borate buffer (pH 9.2) 5 mm per liter, exposed to H<sub>2</sub>S for 4 minutes, NaOH then added to 87 mm per liter, no change on adding Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>; Curve 4, reduced hemochromogen. Total pigment 0.101 mm per liter, borate buffer (pH 9.2) 5 mm per liter, exposed to H<sub>2</sub>S for 4 minutes, pyridine added to 50 per cent, no change on adding Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>; Curve 5, SMHb. Total pigment 0.151 mm per liter, transformed to MHb by addition of K<sub>3</sub>Fe(CN)<sub>6</sub>, 0.158 mm per liter, phosphate buffer (pH 5.9) 7 mm per liter. The acid MHb was exposed to H<sub>2</sub>S for 5 seconds. An identical spectrum was obtained from MHbCN by treatment with H<sub>2</sub>S.

Reduced hemoglobin was not converted to SHb upon exposure to  $\text{H}_2\text{S}$ , unless  $\text{O}_2$  was available (Fig. 5, Curves 1 and 2). Mixtures of Hb + SHb were converted to typical reduced hemochromogens (11) by the addition of pyridine or alkali in excess (Fig. 5, Curves 3 and 4). Conversion to reduced hemochromogen suggests that our preparations of SHb are in a reduced condition.

In confirmation of Keilin's experiments (12) we have found that a characteristic pigment, which may be designated as sulfmethemoglobin (SMHb), was produced when MHb was exposed to  $\text{H}_2\text{S}$  (Fig. 5, Curve 5). In these experiments it was important to avoid any excess of  $\text{K}_3\text{Fe}(\text{CN})_6$  to insure against the precipitation of pigmented material which otherwise occurred. SMHb, in contrast to SHb (see Curve 3, Fig. 3), could be converted to its parent pigment. Upon exposure to air, reconversion of SMHb to MHb took place; the MHb so formed was convertible to typical MHbCN upon the addition of KCN and to typical Hb upon the addition of  $\text{Na}_2\text{S}_2\text{O}_4$ .

We have not found a suitable explanation for the need of  $\text{O}_2$  in the production of SHb from Hb. The formation of SHb did not occur in solutions of  $\text{HbO}_2$  exposed to  $\text{SO}_2$ .

#### SUMMARY

The absorption spectra of  $\text{HbO}_2$ , Hb,  $\text{HbCO}$ , and MHbCN yielded by solutions prepared from hemolyzed washed erythrocytes have been presented. Such solutions have been found preferable for precise spectrophotometric analysis rather than corresponding preparations from hemolyzed whole blood.

The absorption spectrum of HbNO was obtained under conditions which excluded the presence of  $\text{O}_2$ . The conversion of this pigment to MHb has been demonstrated. HbNO is probably a relatively stable analogue of  $\text{HbO}_2$ .

Data have been presented which indicate that the absorption spectrum of pure SHb probably has not been described heretofore. The absorption curve of this pigment has been extrapolated from our data upon mixtures of Hb + SHb. For the first time the quantitative estimation of SHb in the blood of a patient with clinical sulfhemoglobinemia has been accomplished. SHb was formed from Hb only in the presence of oxygen. The pigment was not convertible to Hb or MHbCN, but was readily changed

into typical hemochromogens. Solutions of Hb increase in acidity upon exposure to  $\text{H}_2\text{S}$ .

SMHb, a characteristic pigment described by Keilin (12), was obtained from MHb by treatment with  $\text{H}_2\text{S}$ . A pigment yielding the spectrum of SMHb was obtained also by the exposure of MHbCN to  $\text{H}_2\text{S}$ .

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## SPECTROPHOTOMETRIC STUDIES

### III. METHEMOGLOBIN\*

By J. HAROLD AUSTIN AND DAVID L. DRABKIN

*(From the John Herr Musser Department of Research Medicine and the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia)*

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Much of the important literature upon methemoglobin has been discussed briefly by Peters and Van Slyke (1) up to 1928 and by Maurer (2) to 1930. Conant (3) has recently reviewed those aspects of the methemoglobin problem in which he and his associates have been especially interested. Other papers directly related to our studies will be referred to in discussing our results.

The present study is concerned with spectrophotometric measurements on methemoglobin produced by oxidizing with  $K_3Fe(CN)_6$ ,  $NaNO_2$ , or quinone, the oxyhemoglobin of solutions prepared by hemolyzing washed dog erythrocytes. We have obtained absorption constants for methemoglobin at known values for pH and ionic strength. The curve of transition from the acid to alkaline form is shown. The rate and extent of oxidation as influenced by the kind and amount of the various oxidants used is reported. A method is described for estimating spectrophotometrically the proportions of two pigments in a mixture by the use of a summation of the change in the extinction coefficients at any desired number of selected wave-lengths.

#### *Methods*

As noted in our previous paper (4) concentration of hemoglobin is expressed in mm per liter, where 1 mole signifies 1 mole of porphyrin, equivalent to 1 gm. atom of Fe or 1 mole of  $O_2$  ca-

\* A preliminary report of this study appeared in the Proceedings of the American Society of Biological Chemists (Drabkin, D L, and Austin, J H, *J. Biol. Chem.*, 105, xxiii (1934)).

capacity. As absorption constants we employ  $\epsilon$  ( $c = 1$  mm per liter,  $d = 1$  cm).

A solution of hemolyzed, washed erythrocytes from the dog was prepared as previously described (4). From this solution were prepared quantitatively the various experimental solutions and a solution of MHbCN (4) for determining the concentration of total pigment. The MHbCN solution was read spectrophotometrically at  $\lambda$  540, 545, and 551  $m\mu$  for which our values for  $\epsilon$  ( $c = 1$  mm per liter,  $d = 1$  cm) are 11.5, 11.5, and 11.1 respectively. The absorption in the visible spectrum by  $K_3Fe(CN)_6$  and  $K_4Fe(CN)_6$ , in the concentrations used in our experiments, is entirely negligible (4) and required no correction.

This method of measuring the total concentration of pigment is superior to the methods based upon the extinction coefficient of methemoglobin, MHb, itself at certain wave-lengths, such as those at which the absorption is independent of pH. Haurowitz (5) proposed the measurement of the concentration of MHb based on its absorption at  $\lambda$  623  $m\mu$ , the wave-length at which in his data the extinction coefficients of the acid and alkaline forms coincided. With the method he proposed, one is dependent on a reading at a single wave-length; the error of reading in the red region of the spectrum, moreover, is relatively large and the specific absorption of MHb at  $\lambda$  623  $m\mu$  is only one-third that of MHbCN between  $\lambda$  540 and 551  $m\mu$ . The constants reported by Haurowitz are based, furthermore, on a single crystalline preparation of MHb. Whatever advantages there may be for certain purposes in using crystalline preparations, there can be little doubt that, for establishing constants, preparations of the sort we have employed possess the advantage of greater reproducibility.

It seems to us important in describing such studies as we are reporting on hemoglobin derivatives to specify as completely as possible the composition of the solutions employed and the time relations involved in their preparation. The ionic strength, pH, the order in which reagents are added (in some instances), and the time elapsing at various stages of the preparation, may affect the final result. We have called attention to the importance of time relations (6) in the case of a dilute oxyhemoglobin solution, the concentration of which was 0.01 mm per liter, and

in our study on sulfhemoglobin (4). The importance of time will be pointed out in this paper in connection with all three oxidants and again in a later paper dealing with hemochromogens.

*Calculation of Relative Proportion of Two Components in a Mixture*

For this purpose we have used a method which permits employing simultaneously extinction coefficients obtained at as many wave-lengths as may be desired, but provides in a simple way that the weight given to the values at any wave-length is proportional to the difference in  $\epsilon$  for the two component forms at that wave-length. This method is in general superior to those based on wave-lengths used singly or in pairs or on ratios between values at two wave-lengths. The method is as follows: Let  $A$  and  $B$  be the two components, and  $M$  the mixture. At each wave-length to be used the difference from  $\epsilon(c = 1 \text{ mm per liter})$  for  $A$  to  $\epsilon(c = 1 \text{ mm per liter})$  for  $B$  is measured; the arithmetical sum of these is determined, and this summation of the total change at these wave-lengths may be designated  $\Sigma_T = \Sigma \Delta \epsilon_A \rightarrow \epsilon_B$ . At the same wave-lengths is measured the difference in the same direction from  $\epsilon(c = 1 \text{ mm per liter})$  for  $A$  to  $\epsilon(c = 1 \text{ mm per liter})$  for any mixture,  $M$ , of the two forms. The sum of these values, the summation of the partial change, may be designated  $\Sigma_p = \Sigma \Delta \epsilon_A \rightarrow \epsilon_M$ . The ratio,  $r = \Sigma_p / \Sigma_T$ , measures the fraction of form  $B$ , and  $1 - r$  gives the fraction of form  $A$  in the mixture. Wave-lengths near the extreme red or blue of the spectrum are less desirable because of the greater error of reading, and those wave-lengths are to be avoided at which, for either component, the slope of the absorption curve is steep.

#### EXPERIMENTAL

*Methemoglobin from Oxidation of Oxyhemoglobin by  $K_3Fe(CN)_6$*

*Effect of pH and Ionic Strength*—An  $HbO_2$  solution was converted to MHb, a molar ratio of  $K_3Fe(CN)_6:HbO_2 = 8.16:1$  being used. This MHb was then buffered with phosphate, borate-HCl, or borate-NaOH to the desired pH, which was measured with a glass electrode. The ionic strength of the buffer and ferricyanide was calculated. The composition of each solution is given in the legend of Fig. 1. What contribution the hemoglobin ion may make to the ionic strength is uncertain. If from the base-binding capacity at pH 9.15 we assume a valence of 6 per mole of oxygen capacity,



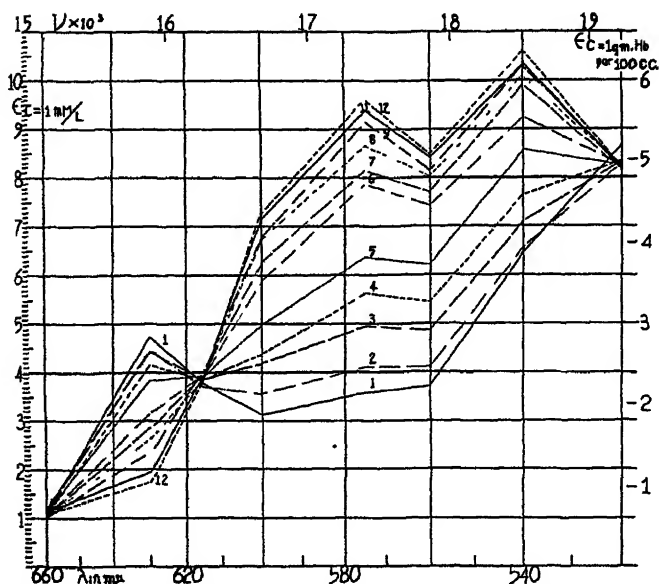


FIG. 1 Curves of methemoglobin from  $K_3Fe(CN)_6$  buffered to various pH values. Molar ratio  $K_3Fe(CN)_6$  total Hb = 8.6; total Hb = 0.107 mm per liter.

Solution No	Buffer	pH	Ionic strength $\mu$
1	Phosphate	6.05	0.032
2	"	7.07	0.052
3	"	7.48	0.062
4	"	7.80	0.070
5	"	8.04	0.077
6	Borate-HCl	8.66	0.022
7	"	8.79	0.029
8	"	9.00	0.043
9	Borate	9.15	0.060
11	Borate-NaOH	9.9	$Na_2B_4O_7$ , 6 mm, NaOH, 8 mm per liter
12	Curve calculated by extrapolation for alkaline MHb (see text)		

then at the concentration of 0.107 mm per liter the hemoglobin would contribute 0.004 to the ionic strength. We infer that it may be neglected for the purposes of our study.

At pH values somewhat less than 6 a tendency for the solution

to become turbid rendered readings unreliable, doubtless because of approach to the isoelectric point; turbidity increased with falling pH until, at about pH 3, return to solution as acid hematin oc-

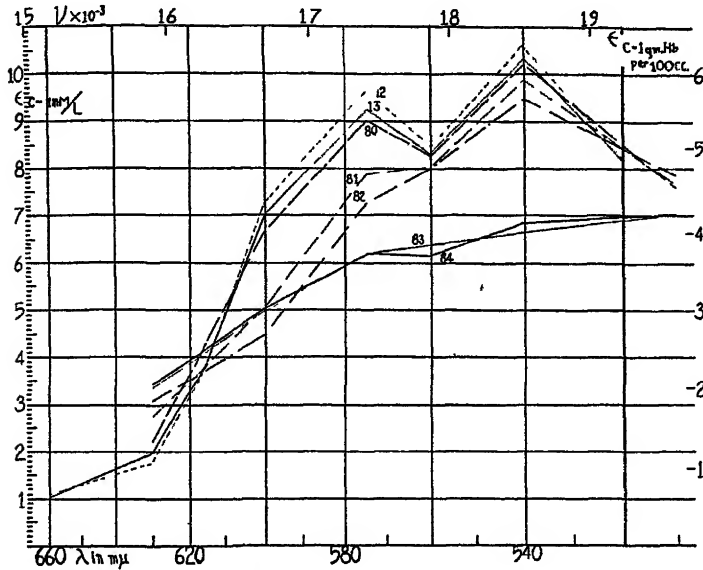


FIG. 2. Curves showing transition from alkaline methemoglobin toward alkaline hematin at pH above 10. Total Hb, Solution 13, 0.107 mm per liter, others 0.207 mm per liter. Molar ratio  $K_3Fe(CN)_6 \cdot Hb$  is 8.6 for Solution 13 and 6.1 for others. Buffers in mm per liter.

Solution No	pH	Buffers
13	11.0	5 $Na_2B_4O_7$ , 10 NaOH
80	10.0	6.3 " 18.8 $Na_2CO_3$ , 47 NaCl
81	11.0	0.7 " 24.3 " 47 "
82	11.5	5 NaOH, 181 NaCl
83	13	100 KOH, 150 NaCl
84	13	100 "
12	Curve calculated by extrapolation for alkaline MHB	

curred. From pH 6.05 to 9.9 a gradual and reversible transition, as shown in Fig. 1, occurred in the character of the absorption.

Our results are in general similar to those of Haurowitz (5), but with significant differences in the constants. Our curves, like

those of Hári (7), show two of the so called "isobestic points" (8) at about  $\lambda$  615 and 520  $m\mu$ . Such isobestic points are characteristic of indicators. Both points in our data are, as compared with those of Haurowitz, shifted toward the blue by 8 to 5  $m\mu$ , and are in this respect in agreement with the data of Hári. This may be ascribed most probably to the less accurate type of apparatus employed by Haurowitz.

At more alkaline values than pH 10 a more irregular and variable alteration in absorption was noted, as shown in Fig. 2, and this tended to be progressive with time. This we interpret as slow alteration toward alkaline hematin at these more alkaline reactions.

The method described above for calculating the proportions of two components was applied to the data of Fig. 1, with the summation ( $\Sigma_p$ ) of the deviations of the values of  $\epsilon$  ( $c = 1$  mm per liter) at  $\lambda$  630, 575, 560, and 540  $m\mu$  from those of Solution 1 at pH 6.05. Nine similar solutions were included, three with phosphate buffers and six with borate-HCl or borate-NaOH. These data are shown as clear circles in Fig. 3, where the ordinate is

$$\frac{\Sigma_p}{\Sigma_T} = \frac{\Sigma_p}{17.80} = \frac{\text{alkaline MHb}}{\text{total MHb}}$$

The data closely approximate the solid curve for the equation

$$\text{pH} = 8.12 + \log \frac{[\text{MHb}]_{\text{alkaline}}}{[\text{MHb}]_{\text{acid}}}$$

Inspection of the "fit" in conjunction with the figures showing ionic strength indicated a systematic deviation correlated with the ionic strength. From the data on six solutions near the mid-point of the curve, it was found that

$$\Delta_{\text{pK}'} = -\alpha \Delta \sqrt{\text{ionic strength}}$$

where  $\alpha$  was approximately 0.6.

Using this value, we corrected the measured pH values to an ionic strength of 0.10. The resulting fit to the theoretical curve is shown in Fig. 3 by the solid dots. The standard deviation of pH in these fifteen observations from the theoretical curve is  $0.039 \pm 0.007$ .

The value of  $\text{pK}'$  in our data is  $8.12 \pm 0.010$ , at an ionic strength

of 0.10, a figure which we consider more precisely determined than those reported by Hartridge (9) and by Haurowitz (5). This example illustrates the accuracy which one may attain, under controlled conditions, in the spectrophotometric estimation of two pigments in a solution, with our method of calculation. The rôle of ionic strength in this change of MHb has not been hitherto

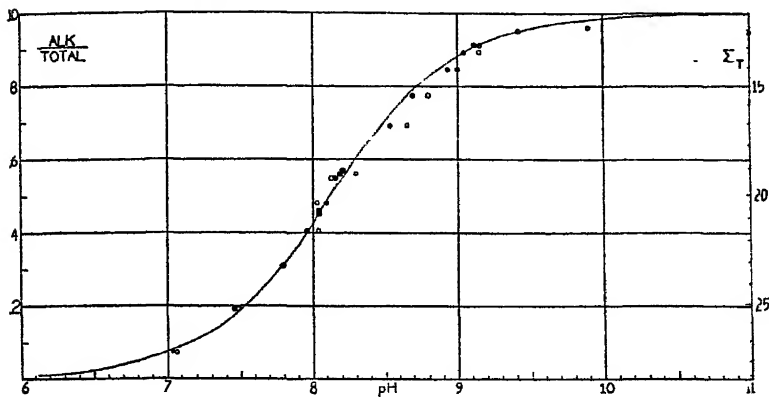


FIG. 3. Partition of acid and alkaline methemoglobin against pH from  $\frac{\Sigma \Delta_{\text{acid}}, \epsilon_{\text{mixture}}}{\Sigma \Delta_{\text{acid}}, \epsilon_{\text{alkaline}}}$  at  $\lambda$  630, 575, 560, and 540  $m\mu$ . Curve for pH = 8.12

+  $\log \frac{\text{MHb}_{\text{alk}}}{1 - \text{MHb}_{\text{alk}}}$ , where  $\text{MHb}_{\text{alk}}$  is the fraction in alkaline form of the total MHb. The clear circles represent observed values at varying ionic strength, the solid dots, observed values with pH corrected to ionic strength of 0.10 (see text). From corrected values,  $\text{pK}' = 8.120 \pm 0.010$ , standard deviation =  $0.039 \pm 0.007$ . The scale at the right is  $\Sigma T = \Sigma \Delta_{\text{HbO}_2, \epsilon} \text{MHb}$  for  $\lambda$  630, 575, 560, and 540  $m\mu$  for the proportion of acid and alkaline forms shown on the scale at the left, or for pH where the curve intersects the ordinate.

demonstrated. From the above and similar data for  $\lambda$  600  $m\mu$  we have obtained Curve 12 of Figs 1 and 2 by extrapolation. We consider this the best approximation to the absorption curve for 100 per cent alkaline MHb, although it is probable that the pigment is too unstable to be obtained unaccompanied by other derivatives, which give the change in absorption indicated in Fig. 2. Between pH 9.2 and 7.0 the change from the acid to the alkaline form of MHb appears to be reversible.

TABLE I  
Titration of HbO<sub>2</sub> with K<sub>3</sub>Fe(CN)<sub>6</sub>

Solution No	Total Hb mM per l	pH	Buffer mM per l	K <sub>3</sub> Fe(CN) <sub>6</sub> (M) Total Hb(M)	$\epsilon$ ( $c = 1$ mM per liter)								$\Sigma \epsilon^*$	$\Sigma \epsilon^\dagger$	$\Sigma \epsilon / \Sigma \epsilon^\dagger$
					$\lambda$ 660 m $\mu$	$\lambda$ 680 m $\mu$	$\lambda$ 690 m $\mu$	$\lambda$ 700 m $\mu$	$\lambda$ 715 m $\mu$	$\lambda$ 725 m $\mu$	$\lambda$ 740 m $\mu$	$\lambda$ 760 m $\mu$	$\lambda$ 780 m $\mu$	$\lambda$ 800 m $\mu$	
25	0.106	7.6†	0	0.24		1.49	1.99	13.16	8.35	13.37	6.48		4.67	25.47	0.18
26	0.106	7.6	0	0.46		2.39	2.67	11.05	7.23	11.75	7.01		10.39	25.47	0.41
27	0.106	7.6	0	0.70		3.19	2.94	9.22	6.76	10.24	7.73		15.00	25.47	0.59
28	0.106	7.6	0	0.93		3.57	3.39	7.01	5.76	8.43	8.48		20.40	25.47	0.80
29	0.106	7.6	0	1.16		4.27	3.79	5.12	5.06	7.45	9.29		24.67	25.47	0.97
30	0.106	7.6	0	2.10		4.87	4.21	4.95	4.99	7.65	9.99		25.31	25.47	1.00
31	0.106	7.6	0	4.20		4.69	4.05	4.99	4.79	7.09	9.20		25.85		
32	0.106	7.6	0	8.40		4.22	4.00	4.77	4.97	7.26	9.20		25.25		
33	0.171	7.15§	13(P)¶	0.35	0.62	1.80	2.09	12.00	7.51	12.82	6.61	7.70	7.50	27.58	0.27
34	0.171	7.14§	13 "	0.69	0.92	3.00	2.72	8.25	5.84	9.71	7.95	8.95	17.23	27.58	0.63
35	0.171	7.13§	13 "	6.9	1.07	4.27	3.54	4.05	4.10	6.57	9.46	9.38	27.58	27.58	1.00
Average HbO <sub>2</sub> ¶						0.45	0.94	15.13	8.73	14.62	5.66		0		
85**	0.107		0	0		0.52		15.31	8.85	14.80			0		
86††	0.214	6.0§	27(P)	0.985		4.47		3.82	3.79	6.21			29.09	29.70	0.98
87	0.214	6.0§	27 "	8.2		4.39		3.46	3.62	6.05			29.70	29.70	1.00
88††	0.107	9.2§	10(B)¶	0.985		1.70		11.54	8.15	11.81			8.64	14.45	0.60
88§§	0.107	9.2§	10 "	0.985		2.07		10.85	8.11	11.20			10.35	14.45	0.72
88	0.107	9.2§	10 "	0.985		2.16		9.70	7.89	10.61			12.40	14.45	0.86
89	0.107	9.2§	10 "	8.2		2.34		8.65	7.81	9.87			14.45	14.45	1.00

- \*  $\Sigma_p = \Sigma \Delta_{\epsilon} \text{HbO}_2$ , solution at  $\lambda$  630, 575, 560, 540  $m\mu$ .  $\epsilon \text{HbO}_2$  for Solutions 25 to 35 is taken from the line "Average  $\text{HbO}_2$ ," for Solutions 86 to 89 from Solution 85
- †  $\Sigma_r$  indicates the value assumed for complete conversion to the oxidized form at pH of the solutions; its source is indicated by the value in bold-faced figures
- ‡ Estimated by interpolating Solutions 30 to 32 in the data of Fig 1
- § Read with the glass electrode
- || P indicates phosphate buffer; B,  $\text{Na}_2\text{B}_4\text{O}_7$
- ¶ Value taken from average of four preparations of oxyhemoglobin in as many experiments
- \*\*  $\text{HbO}_2$  prepared from same hemoglobin solution as Solutions 86 to 89
- †† Read 22 minutes after preparation
- ‡‡ Read 20 minutes after preparation
- §§ Read 80 minutes after preparation
- ||| Read 48 hours after preparation

*Titration of HbO<sub>2</sub> with K<sub>3</sub>Fe(CN)<sub>6</sub>*—Unbuffered HbO<sub>2</sub> solutions were titrated, in contact with air, with K<sub>3</sub>Fe(CN)<sub>6</sub> (see Table I, Solutions 25 to 32, Fig. 4, solid dots) The pH, estimated by interpolation from the curves in Fig. 1 for the pigment converted fully to MHB, was approximately 7.6. A similar titration was carried out on HbO<sub>2</sub> buffered with phosphate at pH 7.14, determined by the glass electrode (Table I, Solutions 33 to 35, Fig. 4,

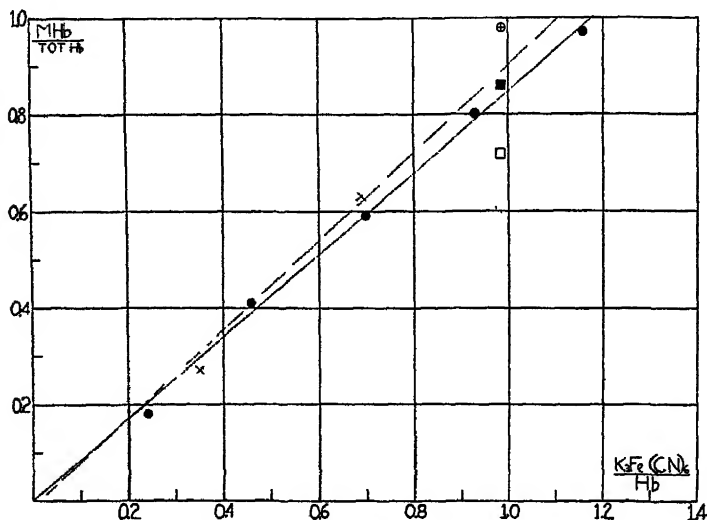


FIG 4 Spectrophotometrically measured mixtures of oxyhemoglobin and methemoglobin plotted against molar ratio of K<sub>3</sub>Fe(CN)<sub>6</sub> total Hb. Circle around a cross represents reading for pH 6.0, read after 22 minutes, crosses, pH 7.14; solid dots, pH 7.6; dotted line square, pH 9.2, read after 20 minutes; clear square, same read after 80 minutes; solid square, same read after 48 hours

crosses). In Fig. 4 are drawn the best straight lines through the experimental points and origin. To two HbO<sub>2</sub> solutions, one (Solution 86) buffered with phosphate to pH 6.0, the other (Solution 88) with borate to pH 9.2, K<sub>3</sub>Fe(CN)<sub>6</sub> was added to give a molar ratio of K<sub>3</sub>Fe(CN)<sub>6</sub>:HbO<sub>2</sub> of 0.985. The extent of conversion plotted against ratio of K<sub>3</sub>Fe(CN)<sub>6</sub>:HbO<sub>2</sub> is shown in Fig. 4. At pH 6.0 with a 1:1 M ratio of K<sub>3</sub>Fe(CN)<sub>6</sub>:HbO<sub>2</sub> the HbO<sub>2</sub> was completely converted within approximately 20 minutes to MHB

As the solution, in contact with air, was made more alkaline, the reaction became much slower and was not completed at a 1:1 m ratio. Meier and his associates (10, 11) reported as high as 98 per cent conversion of  $\text{HbO}_2$  at a 1:1 m ratio with  $\text{K}_3\text{Fe}(\text{CN})_6$  at pH  $-7.0$ , having made their measurements with the Barcroft gasometric method. Their data likewise showed the diminished conversion at higher pH.

*Methemoglobin from Oxyhemoglobin in Presence of  $\text{NaNO}_2$*

*Titration of  $\text{HbO}_2$  with  $\text{NaNO}_2$* —The reaction between  $\text{HbO}_2$  and  $\text{NaNO}_2$  in contact with air, either unbuffered or buffered with phosphate at pH 7.1, was different in some respects, as is shown in Table II and Fig. 5, from that with  $\text{K}_3\text{Fe}(\text{CN})_6$ . There was little or no promptly recognizable conversion to MHb following the addition of the first portions of nitrite. After we had added to a given preparation of  $\text{HbO}_2$  a certain amount of nitrite without recognizable early effect, further addition of nitrite promptly converted the  $\text{HbO}_2$  to MHb or possibly to a mixture of MHb and some other pigment. For about every 0.5 to 0.7 mole of additional nitrite added, 1 mole of  $\text{HbO}_2$  was converted rapidly to MHb. The amount of nitrite which could be added without prompt oxidation of  $\text{HbO}_2$  varied with different preparations, from a molar ratio of 0.24 in the preparation from which Solutions 50 to 54 were made, to a molar ratio of 0.87 in the preparation from which Solutions 43 to 44 were made. When, however, many hours had elapsed after adding only the first portion of nitrite, we found the  $\text{HbO}_2$  eventually converted to MHb at a molar ratio of  $\text{HbO}_2:\text{NaNO}_2 = 1:0.7$  (see Fig. 5, Solutions 46 to 49, made up from the same preparation of  $\text{HbO}_2$  as Solutions 43 to 45).

The reaction of  $\text{HbO}_2$  preparations (in contact with air) with small amounts of nitrite was a slow reaction and probably involved a preliminary reaction of the nitrite with some constituent of the solution which gradually released the oxidant effective in the conversion of the  $\text{HbO}_2$  to MHb. With larger quantities of nitrite a reaction which was relatively rapid made its appearance. In both types of reaction the nitrite effective appeared to convert 1 mole of  $\text{HbO}_2$  for each 0.5 to 0.7 mole of  $\text{NaNO}_2$ .

In Table II the values for  $\Sigma \tau$  may be compared with values at the same pH for MHb derived with  $\text{K}_3\text{Fe}(\text{CN})_6$ . These latter



TABLE II  
Spectrophotometric Readings on Solutions of Oxyhemoglobin Oxidized with  $\text{NaNO}_2$

Solution No	Hb  max per l	$\frac{\text{NaNO}_2(\text{M})}{\text{Hb}(\text{M})}$	pH	PO <sub>2</sub>  max per l	Inter- val before reading  hrs	$\epsilon$ ( $c = 1 \text{ mm per liter}$ )												$\Sigma p^*$	$\Sigma_T^{\dagger}$	$\Sigma_p/\Sigma_T$	$\Sigma_T^*$ Fig 8†	
						$\lambda$ 700 m $\mu$	$\lambda$ 680 m $\mu$	$\lambda$ 660 m $\mu$	$\lambda$ 640 m $\mu$	$\lambda$ 620 m $\mu$	$\lambda$ 600 m $\mu$	$\lambda$ 575 m $\mu$	$\lambda$ 560 m $\mu$	$\lambda$ 540 m $\mu$	$\lambda$ 520 m $\mu$	$\lambda$ 510 m $\mu$	$\lambda$ 500 m $\mu$					$\lambda$ 490 m $\mu$
43-a	0 171	0 91	7 15§	13	2	0 40	0 95	1 62	14	49 8	55 14	25						6 90	1 69	27 3	0 06	27 1
43-b	0 171	0 91	7 15§	13	24	1 10	3 99	3 81	6	21 5	27 8	13						8 80	22 41	27 3	0 82	27 1
44	0 171	1 36	7 15	8	2	0 95	4 16	3 56	4	21 4	32 6	68						9 68	26 98	27 3	0 99	27 1
45	0 171	5 7	7 15	8	2	1 05	4 24	3 54	4	14 4	18 6	63						9 61	27 32	27 3	1 00	27 1
46	0 171	0 11	7 15	6 7	24¶	1 14	2 56	3 16	13	00 8	65 13	50						9 01	5 44	27 3	0 20	27 1
47	0 171	0 23	7 15	6 7	24¶	0 88	2 17	2 54	11	70 8	10 12	48						8 08	7 92	27 3	0 29	27 1
48	0 171	0 46	7 15	6 7	24¶	1 15	3 94	4 12	6	80 7	01 8	45						9 18	19 71	27 3	0 72	27 1
49	0 171	0 68	7 15	13	24¶	1 80	4 81	4 12	5	21 5	35 7	51						10 0	24 77	27 3	0 90	27 1
50	0 105	0 24	7 8**	0		0 99		1 47	15	06 9	09 14	64						0 23	24 8	0 01		
51	0 105	0 47	7 8	0		1 94		2 32	12	19 7	95 12	39						7 44	24 8	0 30		
52	0 105	0 71	7 8	0		3 10		3 66	7	36 5	94 9	15						18 68	24 8	0 75		
53	0 105	0 95	7 8	0		3 92		4 22	5	54 5	31 7	35						23 75	24 8	0 96		
54	0 105	1 42	7 8	0		3 82		4 22	5	22 5	22 7	34						24 07	24 8	0 97		
55	0 105	2 87	7 8	0		4 35		3 91	5	04 5	31 7	26						24 77	24 8	1 00		
56	0 105	4 75	7 8	0		4 64		4 49	5	52 5	78 8	03						23 34				
57	0 105	9 50	7 8	0		4 54		4 31	5	46 5	61 7	76						23 74				
58	0 190	1 05	5 9	25	1	0 62	1 34	4 51	3 95	3 74	5 15	49	7 78	8 78	9 06	9 30		9 00	23 76			29 0
59	0 190	1 05	7 0	25	2	0 87	1 39	4 44	4 04	3 76	5 57	5 72	7 82	8 55	8 95	9 18		8 73	23 36			27 6
60	0 190	4 1	7 0	25	4	0 80	1 39	4 32	3 86	3 87	6 14	6 68	8 72	8 67	8 61	8 45		8 30	20 81	20 8		27 6

\*  $\Sigma_p = \Sigma \Delta \epsilon_{\text{HbO}_2}$ , solution at  $\lambda$  630, 575, 560, 540  $m\mu$  For  $\epsilon_{\text{HbO}_2}$  are used the average HbO<sub>2</sub> values shown in Table I.

†  $\Sigma_p$  indicates the value assumed for complete conversion to the oxidized form; its source is indicated by the value in bold-faced type.

‡ " $\Sigma_p$ , Fig. 3" shows the value found at the same pH on oxidizing with K<sub>3</sub>Fe(CN)<sub>6</sub>. See text for discussion of the difference between the two values for  $\Sigma_p$ .

§ Measured with the glass electrode.

|| Buffer added 2 hours after nitrite and just before the 2 hour reading.

¶ Buffer added 24 hours after nitrite and just before the 24 hour reading.

\*\* In unbuffered Solutions 50 to 57 the pH is not definitely known. Based on the average curve of the fully converted solutions, Nos 54, 55, the pH would be about 7.8.

values may be read from the right-hand scale of Fig. 3 for the corresponding pH and the values so selected are shown in the last column of Table II. In Solution 45, Table II, oxidized with  $\text{NaNO}_2$ , the agreement of  $\Sigma \tau$ , 27.3, with  $\Sigma \tau$ , 27.1, obtained with  $\text{K}_3\text{Fe}(\text{CN})_6$  at pH 7.15 is excellent. In Solution 55 ( $\Sigma \tau$ , 24.8), for which the pH is not accurately known, the comparison cannot be

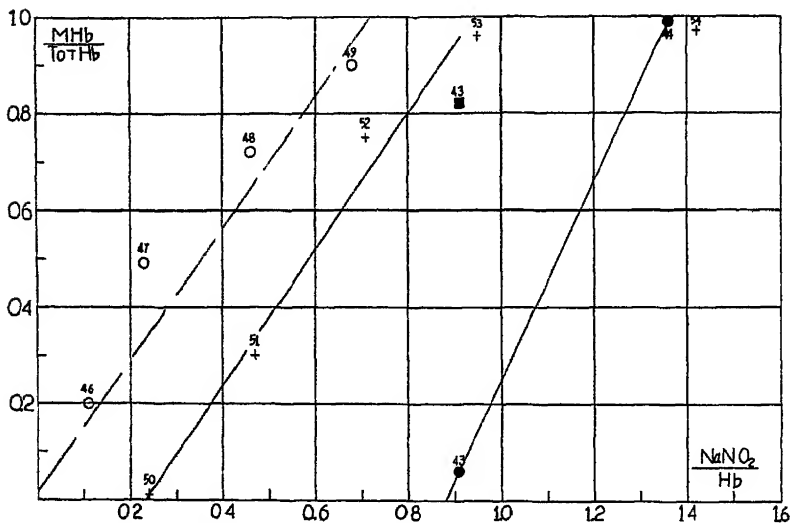


FIG 5 Showing rapid conversion of oxyhemoglobin to methemoglobin by nitrite added in excess of a limit peculiar to each preparation. (For final composition of solutions see Table II; numbers at the points correspond to the solution numbers of Table II) The readings indicated by crosses are for Solutions 50 to 54, in buffered solutions; the solid dots, Solutions 43, 44, standing 2 hours with  $\text{NaNO}_2$  then buffered and read; the solid square, Solution 43, read again after standing 24 hours, following the addition of buffer; the clear circles, Solutions 46 to 49, standing 24 hours with nitrite, then buffered and read at once

made. When the amount of nitrite was increased, as in Solutions 56 and 57, the absorption increased, especially at  $\lambda$  575, 560, and 540  $m\mu$ , with resulting diminution of  $\Sigma \tau$ . The effect was most marked in Solution 60. This elevation of the absorption curve with molar ratios of  $\text{NaNO}_2:\text{HbO}_2$  greater than 4:1 is such as would occur if the solution contained a mixture of MHb and HbNO (4). Meier (12) presented evidence that a mixture of

TABLE III

*Progressive Oxidation after Adding Varying Amounts of Quinone to Oxyhemoglobin Buffered with Phosphate 13 mM per Liter at pH 7.07 Total Pigment 0.120 mM per Liter*

Solution No	Quinone (M) Total Hb (M)	Interval before reading	$\epsilon$ ( $c = 1$ mm per liter)								$\Sigma_p^*$	$\Sigma_T^\dagger$	$\frac{\Sigma_p}{\Sigma_T}$
			$\lambda$ 680	$\lambda$ 630	$\lambda$ 600	$\lambda$ 575	$\lambda$ 560	$\lambda$ 540	$\lambda$ 520				
			$m\mu$	$m\mu$	$m\mu$	$m\mu$	$m\mu$	$m\mu$	$m\mu$				
		hrs											
64-a	1 0	0 33	1 02	1 24	1 60	15 30	8 85	14 92	6 48	0	27 00	0 00	
64-b	1 0	7 50	1 27	1 47	1 75	14 09	8 38	13 90	6 85	2 93	27 00	0 11	
64-c	1 0	21 85	1 47	2 12	2 40	11 39	7 30	11 61	7 21	9 65	27 00	0 36	
64-d	1 0	45 47	1 62	3 22	3 04	8 87	6 36	9 82	7 50	16 00	27 00	0 59	
64-e	1 0	68 50	1 95	3 71	3 31	7 37	5 77	8 51	7 55	19 89	27 00	0 74	
64-f	1 0	92 80	1 95	4 11	3 82	6 21	5 51	7 54	7 81	21 68	27 00	0 80	
65-a	2 5	0 10	1 22	1 37	1 94	15 24	9 51	14 82	7 13	0	26 20	0 00	
65-b	2 5	7 33	1 42	1 69	1 97	13 56	8 25	13 49	7 21	4 59	26 20	0 17	
65-c	2 5	21 85	1 70	3 26	3 09	9 45	7 06	10 53	7 98	14 42	26 20	0 55	
65-d	2 5	45 37	1 92	4 19	3 76	6 25	5 80	8 03	8 15	22 31	26 20	0 85	
65-e	2 5	69 09	1 99	4 31	3 88	5 51	5 34	7 38	7 79	24 28	26 20	0 93	
65-f	2 5	76 51	2 04	4 29	3 90	5 75	5 49	7 46	7 81	23 79	26 20		
65-g	2 5	93 39	2 52	3 82	4 22	7 38	6 90	8 84	8 03	19 01	26 20		
66-a	5 0	0 04	1 35	1 45	1 99	15 35	9 70	14 82	7 21	0	25 00	0 00	
66-b	5 0	7 26	1 69	2 36	2 47	12 10	7 90	12 51	7 50	8 35	25 00	0 33	
66-c	5 0	21 81	1 97	4 26	3 76	7 79	6 75	9 55	8 42	18 67	25 00	0 75	
66-d	5 0	27 35	1 96	4 29	3 91	7 14	6 33	8 81	8 40	20 51	25 00	0 82	
66-e	5 0	45 13	2 05	4 32	3 84	5 37	5 18	7 29	8 11	24 95	25 00	1 00	
66-f	5 0	52 56	2 04	4 32	3 84	5 37	5 25	7 21	8 00	24 99	25 00	1 00	
66-g	5 0	69 73	2 04	4 32	3 88	5 37	5 17	7 30	7 79	24 98	25 00	1 00	
67-a	15 0	0 06	1 57	3 48	3 44	8 86	7 32	10 12	8 74	15 68	20 00	0 78	
67-b	15 0	0 14	1 69	3 88	3 52	7 95	7 10	9 96	9 12	17 37	20 00	0 87	
67-c	15 0	0 29	1 87	3 94	3 51	7 21	6 76	9 40	9 21	19 07	20 00	0 95	
67-d	15 0	0 54	1 87	4 11	3 59	7 10	6 68	9 37	9 21	19 46	20 00	0 97	
67-e	15 0	2 68	1 89	4 13	3 56	7 10	6 76	9 50	9 31	19 27	20 00	0 96	
67-f	15 0	25 95	1 99	4 35	4 06	7 01	7 01	9 45	9 62	19 38	20 00	0 97	

\*  $\Sigma_p = \Sigma \Delta \epsilon_{\text{HbO}_2} \rightarrow \epsilon_{\text{solution}}$  at  $\lambda$  630, 575, 560, 540  $m\mu$ . Value for  $\epsilon_{\text{HbO}_2}$  for Solution 64 taken from Solution 64-a; for Solution 65 from Solution 65-a; for Solutions 66 and 67 from Solution 66-a.

†  $\Sigma_T$  for pH 7.07 from Fig. 3 is 27.5.  $\Sigma_T$  for Solution 66, taken as the average of Solutions 66-f and 66-g = 25.0. The difference 27.5 - 25.0 = 2.5 is attributed to light absorption from quinone. For other solutions this disturbing absorption is assumed proportional to the quinone concentration, giving for Solution 64, 0.5; for No. 65, 1.3; for No. 67, 7.5; hence the  $\Sigma_T$  values shown are calculated.

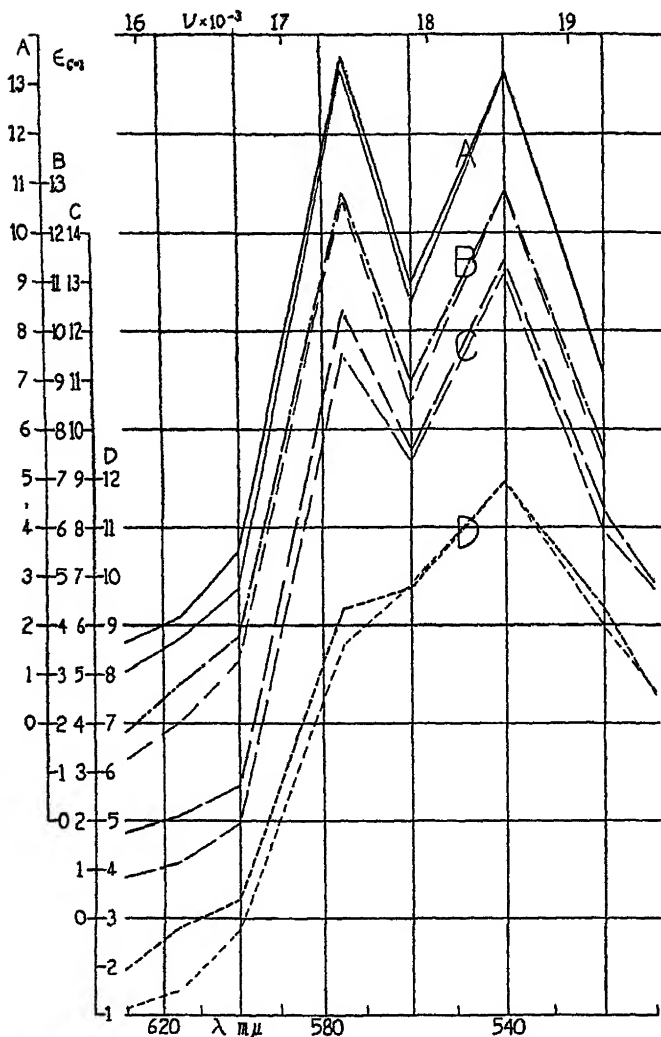


Fig. 6 Conversion of oxyhemoglobin to methemoglobin in dilute solution. Solid line curves, Group A (to be read against "A" scale). heavy line, Solution 70,  $\text{HbO}_2$  0.010 mm per liter in borate buffer 25 mm per liter, pH 9.2, kept at  $4^\circ$  60 hours; light line, calculated curve for 0.316 MHb, pH 9.2, + 0.684  $\text{HbO}_2$ . Dot and dash line curves, Group B (to be read against "B" scale): heavy line, Solution 70, as above, kept at  $4^\circ$  84 hours; light line, calculated curve for 0.402 MHb, pH 9.2, + 0.598  $\text{HbO}_2$ . Dash line curves, Group C (to be read against "C" scale): heavy line, Solution

MHb and HbNO results when  $\text{NaNO}_2$  acts on reduced hemoglobin in the absence of air. Haurowitz (5) interpreted similarly his absorption curves obtained from the derivative produced by the action of  $\text{KNO}_2$  on  $\text{HbO}_2$  exposed to air. Our data upon solutions with excess of  $\text{NaNO}_2$  are not inconsistent with this interpretation.

Meier (12) noted that  $\text{NaNO}_2$  does not convert hemoglobin at weakly alkaline pH. We find likewise that  $\text{NaNO}_2$  added to  $\text{HbO}_2$  buffered with borate to pH 9.2 induced no change in the spectrum of  $\text{HbO}_2$ , at least within a few hours. However, a spectrum essentially identical with that of MHb produced with  $\text{K}_3\text{Fe}(\text{CN})_6$  at pH 9.2 was obtained with nitrite, when this reagent was added in excess to an unbuffered  $\text{HbO}_2$  solution and the resulting derivative subsequently buffered with borate to pH 9.2.

*Methemoglobin from Quinone*—It has been noted (10–12) that complete conversion of  $\text{HbO}_2$  to MHb by means of quinone requires many times the molar ratio of oxidant to hemoglobin.

Hemolyzed cells buffered with phosphate at pH 7.0 were added to flasks containing appropriate amounts of quinone to give molar ratios of  $\text{HbO}_2$ :quinone of 1.0, 2.5, 5.0, and 15.0. Table III shows the extent of the conversion and the time relationships involved. For the three molar ratios, 1.0, 2.5, and 5.0, the rate of change was approximately proportional to the quinone concentration; but the rate at molar ratio 15 was about 800 times the rate at molar ratio 5. The curve obtained with excess of quinone, when the reaction had gone to completion, for example in Solutions 66-e to 66-g, differed somewhat from that obtained from MHb produced with ferricyanide at the same pH, being higher at  $\lambda$  575, 560, and 540  $m\mu$ . At pH 9.2, the curve was higher throughout. With extreme excess of quinone the absorption was increased further still, as may be seen by comparing the values of  $\epsilon$  for Solutions 66-e to 66-g with Solutions 67-c to 67-f. We attribute this effect to absorption by other products derived from the quinone and not to incomplete conversion of  $\text{HbO}_2$  to MHb.

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70 treated after 84 hours at 4° with excess of KCN; light line, calculated curve for 0.471 MHbCN + 0.529  $\text{HbO}_2$ . Dotted line curves, Group D (to be read against "D" scale): heavy line, same as Group C but kept 48 hours longer in the presence of excess KCN at 4°; light line, calculated curve for 0.854 MHbCN + 0.146  $\text{HbO}_2$ .

Solutions of MHB prepared with quinone yielded, after the addition of an excess of KCN, absorption curves closely resembling those of ordinary MHB<sub>CN</sub>, though somewhat higher presumably owing to the same disturbing absorption attributed to quinone products.

*Methemoglobin from Oxyhemoglobin in Dilute Aqueous Solutions, Standing in Contact with Air*

Either in phosphate buffer at pH 7.0 or in borate buffer at pH 9.2 a dilute solution (0.01 mm per liter) of HbO<sub>2</sub>, in contact with

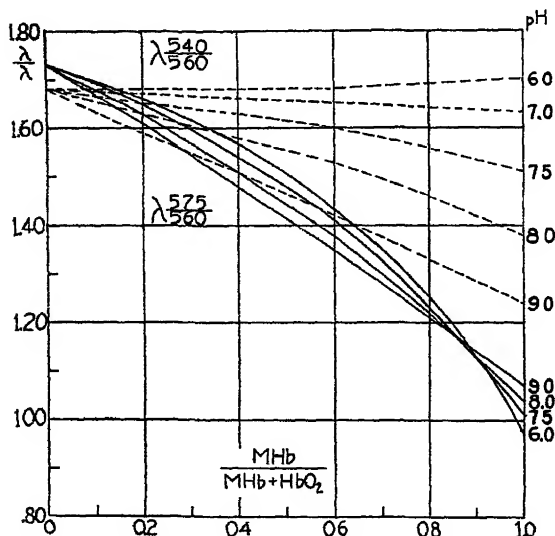


FIG 7. Showing values for ratios of  $\epsilon_{\lambda 575}/\epsilon_{\lambda 560}$  (solid lines) and of  $\epsilon_{\lambda 540}/\epsilon_{\lambda 560}$  (broken lines) at varying mixtures of oxyhemoglobin and methemoglobin and for different pH values  $MHB + HbO_2 = \text{total hemoglobin}$

air, underwent gradual conversion to MHB, as shown for the solution at pH 9.2 in Fig 6 (curves of Groups A and B). The light lines show the calculated curves for those mixtures of MHB and HbO<sub>2</sub>, which correspond most closely with the observed curves. After the solutions had stood for 84 hours KCN was added to them. Upon the addition of the cyanide, the MHB, which had been produced on standing, was promptly converted to MHB<sub>CN</sub> (Fig. 6, Group C). During the next 48 hours the con-

version of  $\text{HbO}_2$  continued at about the same rate as before the addition of KCN. The curves (Fig. 6, Group D) obtained at this time corresponded closely with that calculated for  $0.85 \text{ MHbCN} + 0.15 \text{ HbO}_2$ . Doubt has at times been expressed as to whether the pigment which forms from  $\text{HbO}_2$  in dilute solutions, standing in contact with air, is indeed MHb. In the present experiments there can be little doubt that this was the case. It is also of

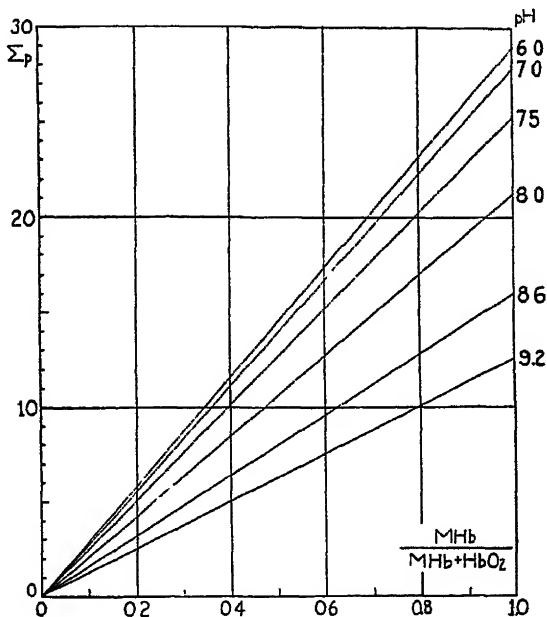


FIG. 8 Values for  $\Sigma \epsilon = \Sigma \Delta \epsilon_{\text{HbO}_2} \rightarrow \epsilon_{\text{mixture}}$  at  $\lambda$  630, 575, 560, and 540  $m\mu$  for varying mixtures of oxyhemoglobin and methemoglobin at varying pH.  $\text{MHb} + \text{HbO}_2 = \text{total hemoglobin}$ .

interest that removal of MHb by converting it to MHbCN had little if any effect on the rate of disappearance of the  $\text{HbO}_2$ . At pH 7.0 the conversion to MHb was about 30 per cent faster than at pH 9.2. These solutions, while standing, were kept at refrigerator temperature, but were not sterile, and bacterial action has not been excluded; no grossly recognizable bacterial decomposition occurred.



*Spectrophotometric Estimation of Methemoglobin in Mixtures with Oxyhemoglobin*

A method which has been advocated for estimating spectrophotometrically the amount of MHb in mixtures with HbO<sub>2</sub> is the use of the ratio of  $\epsilon$  at  $\lambda$  575 or 540  $m\mu$  to  $\epsilon$  at  $\lambda$  560  $m\mu$ . Oddly enough the ratio  $\epsilon_{\lambda 540}/\epsilon_{\lambda 560}$  is often the one recommended. Fig. 7 shows these ratios for various mixtures of HbO<sub>2</sub> and MHb at various pH values. The ratio  $\epsilon_{\lambda 575}/\epsilon_{\lambda 560}$  is obviously preferable, both because the change in this ratio is greater and because it is less influenced by pH. If we assume an error of  $\pm 0.05$  for  $\epsilon$  ( $c = 1$  mm per liter), then the error of these ratios, if pH be known, is about 1 per cent. This corresponds for  $\epsilon_{\lambda 575}/\epsilon_{\lambda 560}$ , as may be seen from Fig. 7, to an error of from 5 to 0.5 parts of MHb per 100 of total pigment, depending on the region of the curve being read. For  $\epsilon_{\lambda 540}/\epsilon_{\lambda 560}$  the error is greater.

Provided the pH be known, the method of choice is the use of  $\Sigma_p/\Sigma_T$  as explained in the text, where

$$\Sigma_p = \Sigma \Delta \begin{matrix} \text{HbO}_2 \\ \epsilon \longrightarrow \epsilon \end{matrix} \text{ mixture}$$

and

$$\Sigma_T = \Sigma \Delta \begin{matrix} \text{HbO}_2 & \text{MHb} \\ \epsilon \longrightarrow \epsilon \end{matrix}$$

at  $\lambda$  630, 575, 560, and 540  $m\mu$  at the pH of the solution. The values for this ratio at varying pH values are shown in Fig. 8. For an error of  $\pm 0.05$  for  $\epsilon$  ( $c = 1$  mm per liter) the error for MHb is only 0.2 to 0.3 part per 100. This method requires measurement of three values or sets of values: (1) the spectrophotometric extinction coefficients of the mixture at  $\lambda$  630, 575, 560, and 540  $m\mu$ ; (2) the pH of the mixture; (3) the concentration of total pigment by the conversion of the pigment to MHbCN, and measurement of its concentration spectrophotometrically.

## SUMMARY

1. Spectrophotometric constants are given for methemoglobin, MHb, derived from washed, hemolyzed dog erythrocytes treated with  $K_3Fe(CN)_6$  at various pH values and at known ionic strengths. From pH 6.0 to pH 9.4 the pigment undergoes conversion from the acid to alkaline form, the conversion conforming closely to the equation

$$\text{pH} = \text{pK}' + \log \frac{\text{MHb}_{\text{alkaline}}}{\text{MHb}_{\text{acid}}}$$

The value for  $\text{pK}'$  at an ionic strength of 0.10 is  $8.120 \pm 0.010$ ; and very roughly

$$\Delta \text{pK}' = -0.6 \Delta \sqrt{\text{ionic strength}}$$

at least below an ionic strength of 0.154.

2. At about pH 9.4, where 95 per cent of methemoglobin is in the alkaline form, an alteration sets in, which is increased at higher pH values and which progresses with time. This alteration appears spectrophotometrically to be in the direction of alkaline hematin. Between pH 7.0 and 9.2, the change between the acid and alkaline form of methemoglobin is reversible.

3. Titration of oxyhemoglobin with  $\text{K}_3\text{Fe}(\text{CN})_6$ , in contact with air, shows that oxyhemoglobin is converted to methemoglobin completely at pH 6.0 within 20 minutes by a mole to mole ratio of reductant to oxidant. As the pH rises an excess of  $\text{K}_3\text{Fe}(\text{CN})_6$  is required to complete the conversion, and for a given ratio of  $\text{K}_3\text{Fe}(\text{CN})_6:\text{HbO}_2$  the reaction proceeds more slowly.

4. On treating our oxyhemoglobin solutions with small amounts of nitrite there is a slow reaction in which the nitrite appears to act only indirectly on the oxyhemoglobin, the rate of this reaction is accelerated at lower pH values. Nitrite added in excess of an amount characteristic for a particular preparation gives immediate conversion to methemoglobin. 1 mole of oxyhemoglobin is converted for every 0.5 to 0.7 mole of  $\text{NaNO}_2$  effective in the reaction. With quantities of nitrite in excess of a molar ratio of 4.1 at pH about 7.15 the resulting absorption curve is higher at  $\lambda$  575, 560, and 540  $m\mu$  than that obtained with  $\text{K}_3\text{Fe}(\text{CN})_6$ . The observed absorption curve is not inconsistent with that which would result from a mixture of methemoglobin and nitric oxide hemoglobin. At pH 9.2,  $\text{NaNO}_2$  did not convert oxyhemoglobin in contact with air to methemoglobin. If methemoglobin be produced with  $\text{NaNO}_2$  in unbuffered solution and then buffered to pH 9.2, it gives a spectrum essentially identical with that of methemoglobin produced with  $\text{K}_3\text{Fe}(\text{CN})_6$  at pH 9.2.

5. There is a slow reaction between oxyhemoglobin and small amounts of quinone in contact with air, but progressing with the

lapse of time to at least 80 per cent conversion at a 1:1 M ratio; a very much more rapid reaction occurs with considerable excess of quinone. The absorption curve of methemoglobin derived with quinone is somewhat higher than that of methemoglobin obtained with ferricyanide; this may be due to by-products. Addition of KCN produces the spectral curve characteristic of cyanmethemoglobin, except that it is likewise somewhat elevated.

6. Oxyhemoglobin in a very dilute solution (0.01 mm per liter) of hemolyzed dog erythrocytes underwent, on standing 84 hours, partial change into a pigment which was proved to be methemoglobin. Addition of KCN converted the methemoglobin which had formed into cyanmethemoglobin, and during a further 48 hours conversion of the oxyhemoglobin continued in the presence of cyanide.

7. Graphs are presented for various mixtures of oxyhemoglobin and methemoglobin at various pH values which show (1) the ratios of  $\epsilon_{\lambda 575 \text{ m}\mu} / \epsilon_{\lambda 560 \text{ m}\mu}$  and  $\epsilon_{\lambda 540 \text{ m}\mu} / \epsilon_{\lambda 560 \text{ m}\mu}$  and (2) the summation of changes in what we have defined as  $\Sigma_p / \Sigma_T$  at  $\lambda 630, 575, 560$ , and  $540 \text{ m}\mu$ . The use and advantages of the latter in the quantitative estimation of methemoglobin in a mixture with oxyhemoglobin are discussed.

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## SPECTROPHOTOMETRIC STUDIES

### IV. HEMOCHROMOGENS\*

By DAVID L DRABKIN AND J HAROLD AUSTIN

(From the Department of Physiological Chemistry and the John Herr Musser  
Department of Research Medicine, School of Medicine, University of  
Pennsylvania, Philadelphia)

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Biological interest in hemochromogens has been revived in recent years. Anson and Mirsky (1) have shown that hemochromogens are produced by the union of reduced hemin and a nitrogen-containing compound. From the work of Warburg and Negelein (2) there is some reason to believe that the respiratory ferment (*Atmungsferment*) may have a hemochromogen-like structure, thus giving hemochromogens a direct biological significance. The ease with which hemin unites with both simple and complex nitrogenous substances and the extreme ease with which such derivatives are oxidized raise questions as to the possibility that hemochromogens may play important rôles in the intermediary metabolism of hemoglobin. Reliable quantitative data upon the absorption spectra of hemochromogens are needed. The values for the absorption maxima presented by Haurowitz (3) are significantly low, indicating probably incomplete conversion to hemochromogen. Denés (4), on the other hand, has published some apparently trustworthy data upon reduced globin hemochromogen prepared from crystalline horse hemoglobin.

Our data have led us to adopt a use of terms which more exactly fit the results than certain names now in use. The terms and their definitions follow.

*Oxidized Hemin*—This term will be used for solutions of crystal-

\* A preliminary report upon this work was presented before the meeting of the American Society of Biological Chemists at Cincinnati, and an abstract of the report has appeared (*J Biol Chem*, 100, xxvi (1933))

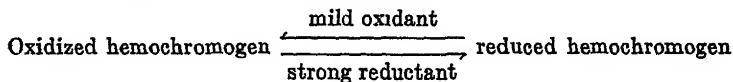
## 90 Spectrophotometry of Hemochromogens

line Cl-hemin, prepared from blood cells by a slight modification of the acetic acid-sodium chloride procedure

*Reduced Hemin*—This term will be applied to solutions of oxidized hemin which have been treated with an excess of  $\text{Na}_2\text{S}_2\text{O}_4$ .

*Reduced Hemochromogen*—This term, in place of the older *hemochromogen*, will be used for solutions containing the product of union of reduced hemin and a nitrogenous substance. The same term will be used for solutions which have absorption spectra which closely resemble that given by a solution containing the compound of reduced hemin and a nitrogenous substance. The absorption spectra of reduced hemochromogens are so characteristic as to be unmistakable. For convenience in discussion the term may be amplified to include the nitrogenous substance, for example, *reduced pyridine hemochromogen*.

*Oxidized Hemochromogen*—This term will be applied to solutions which yield fairly characteristic spectra, and which upon the addition of a strong reducing agent,  $\text{Na}_2\text{S}_2\text{O}_4$ , are at once converted into typical reduced hemochromogens. Such solutions are obtained, for example, when reduced hemochromogens are exposed to air or mild oxidation. Between the reduced and oxidized forms of hemochromogen the following relation exists.



The term *oxidized hemochromogen* seems called for because certain older usages are not unambiguous. Keilin (5) has used the term *parahematin* in place of the older term *cathemoglobin* (6) to describe analogous pigments. The term hematin, as used by Anson and Mirsky (1) does not appear to fit the situation, since oxidized hemochromogens are spectroscopically distinct from hematins. Like Keilin's parahematin they may be converted to hematins, but only after the action of strong acid or alkali.

### Methods

The various procedures used have been described previously (7), except for slight changes necessitated by the problem in hand. For example, the light absorption of reduced hemochromogens is such that the determinations had to be carried out upon solutions approximately one-half the concentration usually employed for

hemoglobin. The  $\epsilon$  values as heretofore are for 1 cm. of depth and for a concentration of 1 mm (of Fe) per liter. Upon this basis the ratio of equivalent weights of pure chlorohemin (8) and hemoglobin (9) is 0.335 per cent Fe/8.57 per cent Fe = 1/25.6. Since the crystalline hemin prepared by us contained slightly less iron, the actual conversion factor was 1/25.4, or 1 gm. of chlorohemin = 1.52 mm of porphyrin.

#### *Preparation of Chlorohemin*

The method of Nencki and Zaleski (10) was modified mainly by the use of washed blood cells instead of whole blood. The diluting effect of serum was thereby avoided as well as the presence of serum proteins and lipids. Since the procedure resulted in a high yield of a uniform preparation of large, beautiful crystals, it is briefly outlined. Thoroughly washed dog blood cells (representing 500 cc. of fresh, defibrinated blood) were delivered from a separatory funnel in a steady, broken stream into approximately 6 volumes of hot glacial acetic acid saturated with NaCl. During this procedure fairly vigorous stirring was maintained, the tip of the funnel being about 6 inches above the surface of the acid. The temperature of the acid was kept at 90–87° by continued heating in a water bath. No precipitate formed on the stirring rod or beaker, so that filtration was unnecessary (whereas it is in the older procedures).

The highly colored solution was maintained at a temperature of 87–90° for 25 minutes after completing the addition of cells, vigorous stirring being continued. The solution was then permitted to cool gradually to room temperature. The first crop of crystals formed after standing at room temperature overnight and was obtained by siphoning off the supernatant colored fluid. After several days of standing the solution yielded a second, almost equal crop of crystals. Each batch of crystals was treated further as follows: They were washed by standing under distilled water overnight. The water was siphoned off. After two further, rapid washings with small quantities of ice-cold distilled water, which was siphoned off, the crystals were permitted to stand under a small volume (a little less than the original volume of cells used) of 65 per cent alcohol for 2 days. This procedure was repeated with fresh 65 per cent alcohol. After the alcohol was siphoned off,

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the crystals were dried for 3 days at 38°, collected upon watch-glasses, and further dried in vacuum desiccators at 38°. A small amount of crystals may be recovered from the alcohol washings. The total yield of crystals was 64 per cent of the theoretical. The iron content, by the Kennedy method (11), was 8.50 per cent for the first crop and 8.48 per cent for the second crop of crystals.

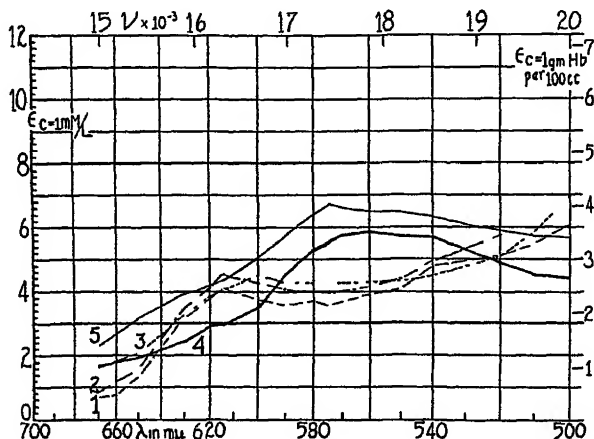


FIG. 1 Oxidized and reduced hemin. Curve 1 represents oxidized hemin, 0.152 mm per liter of hemin in 0.02 M borate buffer, pH about 12, Curve 2, oxidized hemin, 0.061 mm per liter of hemin in 0.02 M borate buffer, pH about 12, Curve 3, oxidized hemin, 0.051 mm per liter of hemin in 0.03 M borate buffer, pH about 10, Curve 4, reduced hemin, 0.024 mm per liter of hemin in 0.02 M borate buffer, pH about 12, with approximately 4 mm per liter of  $\text{Na}_2\text{S}_2\text{O}_4$ ; Curve 5, reduced hemin, 0.020 mm per liter of hemin in 0.02 M borate buffer, pH about 10, with approximately 4 mm per liter of  $\text{Na}_2\text{S}_2\text{O}_4$ .

### Results

The absorption curves of oxidized and reduced hemins in alkaline solution (Fig. 1), though characteristic, are relatively flat. Under the conditions of our experiments the spectra of the oxidized form were more reproducible. pH had no recognized influence on absorption.

Fig. 2 presents the spectra given by solutions of oxidized hemin to which pure anhydrous pyridine was added. In contrast with the ease with which pyridine (in the presence of air) produced oxidized hemochromogen from  $\text{HbO}_2$  and the ease with which it combined with reduced hemin, little evidence of reaction was

obtained when pyridine alone was added to oxidized hemin. When both pyridine and KCN were added to this pigment, however, the solution yielded a spectrum characteristic, as will be seen from later experiments, of oxidized hemochromogen (Curve 3, Fig 2). Qualitatively the spectra of most oxidized hemochromo-

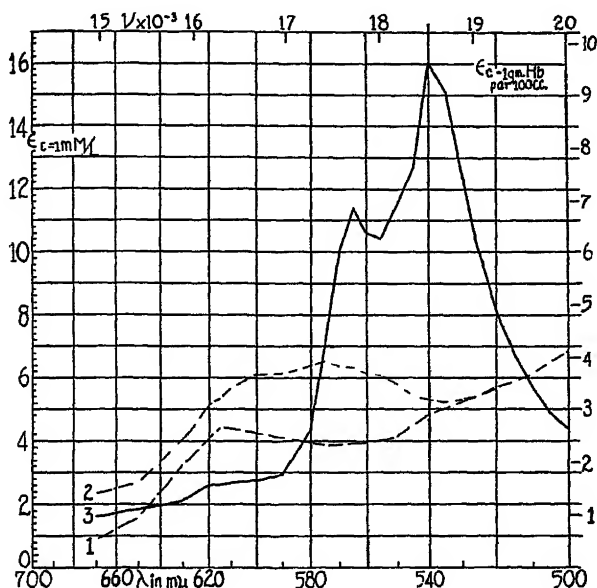


FIG 2 Oxidized hemin  $\rightarrow$  oxidized hemochromogen. Curve 1 represents oxidized hemin, 0.061 mm per liter of hemin in 0.019 M borate buffer, pH about 12, with 6 per cent of pyridine (760 mm per liter); Curve 2, oxidized hemin + hemochromogen(?), 0.018 mm per liter of hemin in 0.019 M borate buffer, pH about 12, with 2.8 per cent of pyridine (355 mm per liter); Curve 3, oxidized hemochromogen from hemin, 0.024 mm per liter of hemin in 0.015 M borate buffer, pH about 12, with 2.4 per cent of pyridine (304 mm per liter) and 0.003 M KCN

gens might be mistaken for that of alkaline methemoglobin (12), although the resemblance is but superficial

Reduced hemin (or oxidized hemin in the presence of a strong reducing agent) was completely converted into reduced pyridine hemochromogen *only when a sufficient excess of the nitrogenous base was added* to the hemin solution (Fig 3) In the preparation of



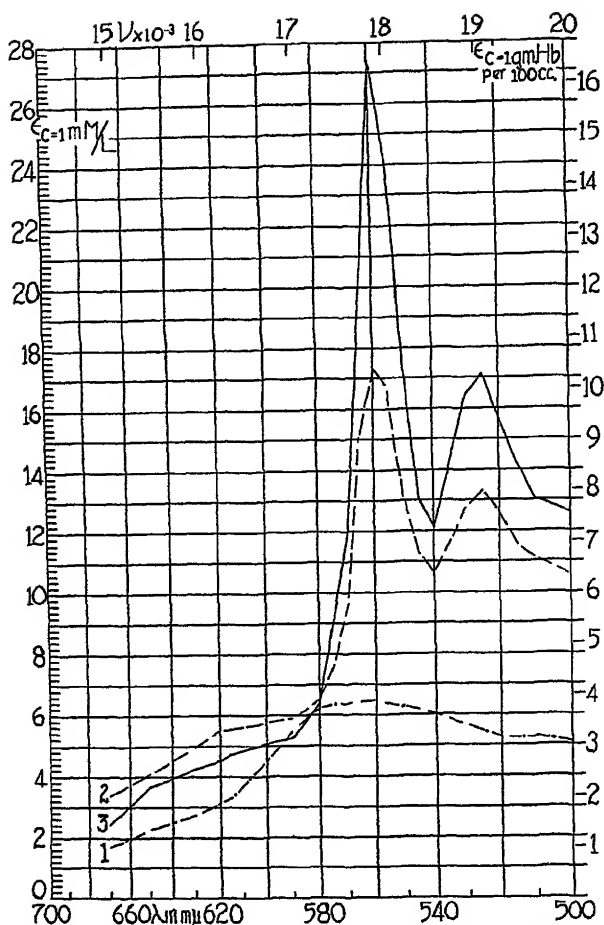


FIG. 3. Reduced hemin  $\rightarrow$  reduced pyridine hemochromogen. Curve 1 represents reduced hemin, 0.0243 mm per liter of hemin in 0.020 M borate buffer, pH about 12, with 2.4 per cent of pyridine (304 mm per liter; M pyridine to M hemin = 12,000) and approximately 4 mm per liter of  $\text{Na}_2\text{S}_2\text{O}_4$ ; Curve 2, partial conversion of reduced hemin to reduced pyridine hemochromogen, 0.0080 mm per liter of hemin in 0.028 M borate buffer, pH about 10, with 0.64 per cent of pyridine (81 mm per liter; M pyridine to M hemin = 10,000) and approximately 4 mm per liter of  $\text{Na}_2\text{S}_2\text{O}_4$ . Prepared by diluting 2.5 with buffer a reduced solution containing 1.6 per cent of pyridine; Curve 3, complete conversion of reduced hemin to reduced pyridine hemochromogen, 0.0095 mm per liter of hemin in 0.019 M borate buffer, pH about 12, with 1.72 per cent of pyridine (218 mm per liter; M pyridine to M hemin = 24,000) and approximately 4 mm per liter of  $\text{Na}_2\text{S}_2\text{O}_4$ . Prepared by adding pyridine from original concentration of 2.4 per cent (Curve 1) to 4.3 per cent, then diluting 2.5 with buffer.

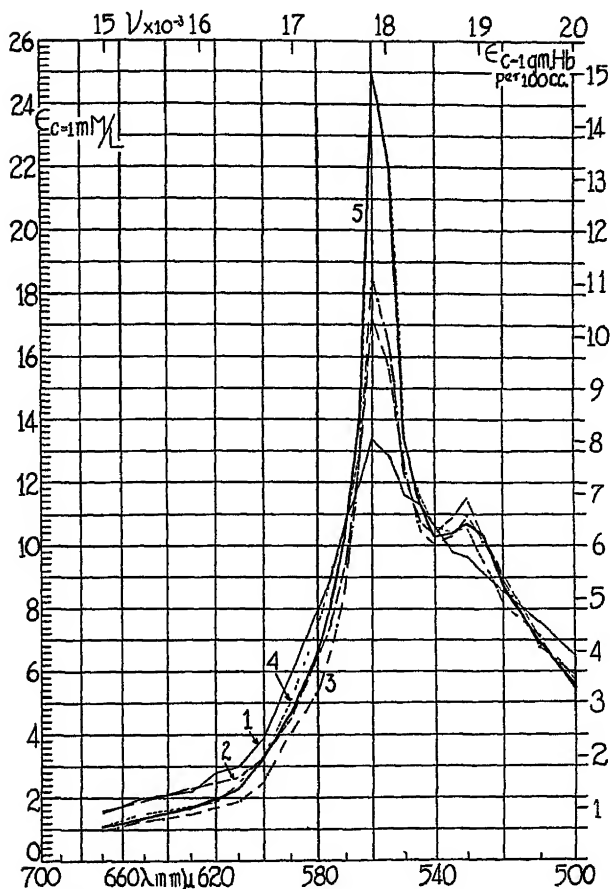


FIG 4. Hemoglobin  $\rightarrow$  reduced globin hemochromogen. Curve 1 represents partial conversion of Hb to reduced globin hemochromogen, 0.12 mm per liter of  $\text{HbO}_2$ , reduced to Hb by evacuation with  $\text{N}_2$ . Read 5 minutes after addition of NaOH to concentration of 0.009 M, pH about 11.8; Curve 2, partial conversion of Hb to reduced globin hemochromogen. Same solution as for Curve 1, read 30 minutes after addition of NaOH; Curve 3, partial conversion of Hb to reduced globin hemochromogen, 0.10 mm per liter of  $\text{HbO}_2$ , reduced to Hb by evacuation with  $\text{N}_2$ . Read 35 minutes after addition of NaOH to concentration of 0.010 M, pH about 11.9; Curve 4, reduced globin hemochromogen (complete conversion from Hb), 0.23 mm per liter of  $\text{HbO}_2$ , reduced to Hb by evacuation with  $\text{N}_2$ . Read 40 minutes after addition of NaOH to concentration of 0.008 M, pH about 11.6; Curve 5, reduced globin hemochromogen from Hb. Same solution as for Curve 4, read 80 minutes after addition of NaOH.

reduced hemochromogens the *element of time* also appeared of importance. Fig 4 depicts the spectra obtained by following the gradual conversion of reduced hemoglobin (reduced by evacuation in  $N_2$  and  $CO_2$ ) into reduced globin hemochromogen after the addition of NaOH. Under our conditions total conversion was obtained only after 40 minutes. The factor of time has apparently been overlooked by most investigators except Denés (4), who allowed  $\frac{1}{2}$  hour for the development of the spectrum of globin hemochromogen. The spectra depicted in Fig 4 are probably a

TABLE I  
Absorption Constants of Hemochromogens

Pigment	$\alpha$ band		Trough		$\beta$ band		$\alpha$ hemochromogen $\alpha$ $HbO_2$ Ratio,	Ratio, $\alpha$ trough	Ratio, $\beta$ trough
	$m\mu$	$\epsilon^*$	$m\mu$	$\epsilon^*$	$m\mu$	$\epsilon^*$			
$HbO_2$	575	15 13	560	8 73	540	14 62		1 73	1 68
Reduced pyridine hemochromogen from hemin	560	27 43	540	12 13	525	17 17	1 81	2 26	1 42
Reduced pyridine hemochromogen from Hb	555	26 98	540	10 73	525	15 13	1 78	2 51	1 41
Reduced globin hemochromogen from Hb	560	25 00	540	10 31	530	10 72	1 65	2 43	1 04
Oxidized globin hemochromogen from Hb	575	9 10	565	8 33	545	10 52	0 60	1 09	1 26
Oxidized pyridine hemochromogen from Hb	555	9 50	550	9 45	530	11 38	0 63	1 01	1 20

\*  $\epsilon$  ( $c = 1$  mm per liter)

quantitative expression of a reaction analogous to the old von Kruger reaction (13), in which interest has been revived (14) since different rates of change of hemoglobin after adding NaOH have been used as evidence for the existence of more than one form of hemoglobin in a species

The remarkable character of the spectrum of reduced hemochromogen is best brought out by the ratio of its peak in the green to that of the so called  $\alpha$  band of oxyhemoglobin, as well as a comparison of the ratios of these peaks to the respective troughs

between the two bands (Table I) There can be no doubt that Haurowitz (3) was dealing for some reason with incompletely developed spectra of hemochromogens, since it may be calculated from his data that the maximum of absorption attained in most of his experiments was more than 25 per cent below our figures.

Reduced hemoglobin, prepared in the usual manner by evacuation of a solution of oxyhemoglobin in a tonometer in the presence

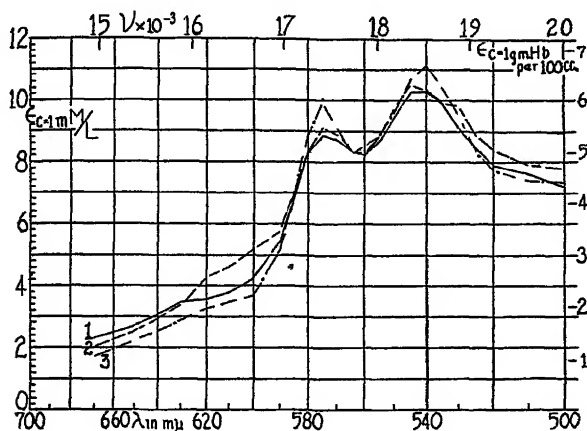


FIG. 5. Reduced globin hemochromogen  $\rightarrow$  oxidized globin hemochromogen. Curve 1 represents oxidized globin hemochromogen from Hb, 0.18 mm per liter of  $\text{HbO}_2$ , partially reduced to Hb by evacuation with  $\text{N}_2$ , NaOH added to concentration of 0.008 M, pH about 12. Read after exposure to air; Curve 2, oxidized globin hemochromogen from Hb, 0.12 mm per liter of  $\text{HbO}_2$ , reduced to Hb by evacuation with  $\text{N}_2$ , NaOH added to concentration of 0.009 M, pH about 12. Read after exposure to air; Curve 3, oxidized globin hemochromogen from Hb, 0.23 mm per liter of  $\text{HbO}_2$ , reduced to Hb by evacuation with  $\text{N}_2$ , NaOH added to concentration of 0.008 M, pH about 12. Read after exposure to air.

of  $\text{N}_2$  and  $\text{CO}_2$ , was mixed in the tonometer with a solution of NaOH also previously freed of oxygen by evacuation. The typical reduced globin hemochromogen solution so formed (see Fig. 4) was exposed to air. There was a rapid change in color from the brick vermillion characteristic of the reduced hemochromogen to a deep red. The qualitative change in spectrum was such that one might suppose that oxyhemoglobin had been regenerated—an impression created by a shift of the bands toward

the red region and a relative equalization of the strength of the two bands. The spectrum obtained, which we consider typical of an oxidized hemochromogen (Fig. 5), is actually very different from that of oxyhemoglobin. It resembles somewhat the spectrum of the alkaline form of methemoglobin (12). A comparison of the ratios at the peaks and trough strikingly bring out the spectral differences in these various pigments (Table I).

Figs. 6 to 8 depict the spectra in a series of experiments which may be considered as a unit. Theoretical considerations, which need not be discussed here, led us to attempt to prepare hemochromogens by the direct addition of anhydrous pyridine to solutions of Hb and HbO<sub>2</sub>.

When pyridine in large excess was added to hemoglobin solutions (reduced by evacuation in a tonometer with N<sub>2</sub> and CO<sub>2</sub> or by means of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>) the spectra gave evidence of the production of typical reduced hemochromogens (Fig. 6). The spectrum very closely resembled, but was not identical (position of the  $\alpha$  band) with that obtained by the addition of pyridine to reduced hemin. Upon exposure to air the solution rapidly changed color and yielded a spectrum typical of oxidized hemochromogen (Fig. 6). The same spectrum of oxidized hemochromogen was obtained directly by the addition of pyridine to a solution of HbO<sub>2</sub> in an open vessel. It is of interest to note that upon the addition of pyridine to reduced hemoglobin a characteristic brick vermilion precipitate was formed when the concentration of pyridine was 8 per cent; upon further addition of pyridine to a concentration of 40 per cent the precipitate dissolved. The spectra of reduced hemochromogens were from solutions with the latter high concentration of pyridine. There is some reason to believe, however, that conversion to reduced hemochromogen had taken place at the lower (8 per cent) concentration. This is inferred from the fact that upon the addition of pyridine to an exposed solution of oxyhemoglobin the change from HbO<sub>2</sub> to oxidized hemochromogen occurred after only 8 to 10 per cent of the nitrogenous base had been added. This change could be followed spectroscopically, since no precipitate formed under these conditions.

The reduced hemochromogen solution, prepared by the addition of oxygen-free pyridine to a reduced hemoglobin solution in a tonometer, was equilibrated thoroughly with pure CO. A definite

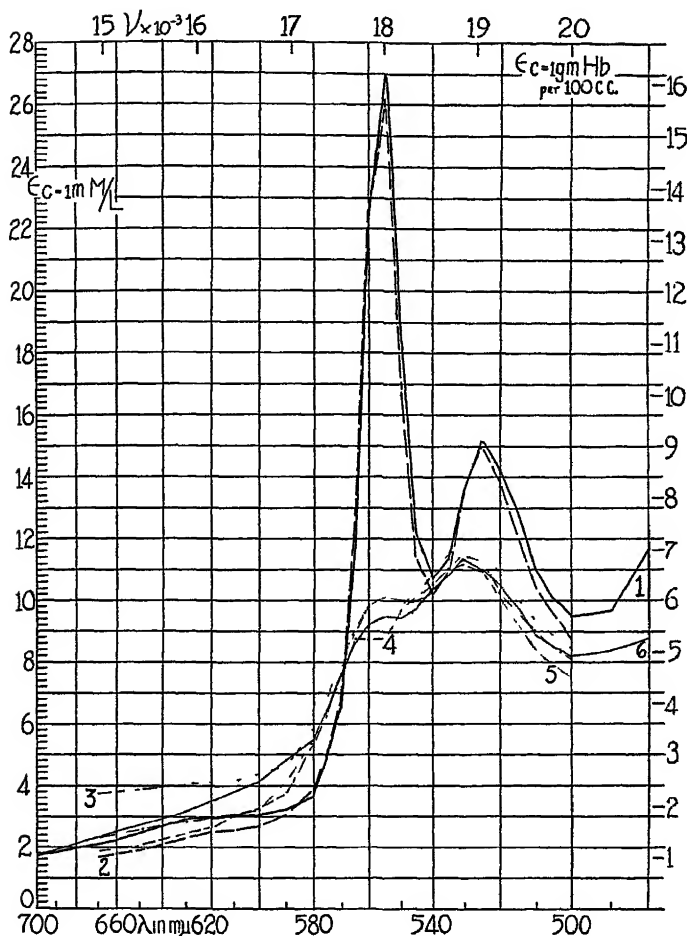


FIG 6. Reduced and oxidized pyridine hemochromogens prepared by addition of pyridine to hemoglobin. Curve 1 represents reduced pyridine hemochromogen from Hb, 0.06 mm per liter of HbO<sub>2</sub>, buffered to pH 8.0 with 0.007 M phosphate buffer, reduced to Hb by addition of 0.004 M Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, pyridine added to concentration of 40 per cent, Curve 2, reduced pyridine hemochromogen from Hb, 0.12 mm per liter of HbO<sub>2</sub>, buffered to pH 8.5 with 0.008 M phosphate buffer, reduced to Hb by evacuation with N<sub>2</sub>, pyridine added to concentration of 41 per cent; Curves 3 to 6, oxidized pyridine hemochromogen from Hb, concentration of Hb 0.05 to 0.12 mm per liter, buffered to pH 7.0 to 8.6 (glass electrode determination) with 0.007 to 0.013 M phosphate. All four solutions with 40 per cent pyridine. In two of the solutions pyridine was added to reduced Hb, then reoxygenated. In the others pyridine was added to HbO<sub>2</sub> in the presence of air.

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change of color occurred and the resulting solution had a spectrum which fairly closely resembled that of carboxyhemoglobin (Fig. 7). However, this was a completely reversible process, the spectrum of reduced hemochromogen being restored after evacuation with  $N_2$ . Under the conditions of our experiments, the reduced pyridine hemochromogen prepared from hemin did not react with CO, although such a reaction has been described (1). It is to be noted that illuminating gas, due to impurities or improper experimental conditions, merely produced oxidation of the hemochromogen (Fig. 7).

Upon the addition of  $K_3Fe(CN)_6$  to the oxidized hemochromogen prepared by the exposure of the reduced pyridine hemochromogen (from hemoglobin) to air no further change in spectrum occurred (Fig. 8). This experiment definitely indicates that we were dealing with an oxidized pigment and that the terminology *oxidized hemochromogen* is justifiable.<sup>1</sup> When, however, this oxidized hemochromogen, without previous treatment with  $K_3Fe(CN)_6$  was treated with KCN, the solution yielded a spectrum very similar to that of cyanhemoglobin. The difference in level of the typical cyanhemoglobin spectrum and that in the experiment may be accounted for by change in concentration during the previous treatment of the solution in the tonometer. It may be added that this reaction with KCN was not given by our preparation of oxidized globin hemochromogen (Fig. 5).

Our data do not permit an unequivocal explanation of the behavior with CO and KCN of the hemochromogen produced by the treatment of hemoglobin with pyridine. These hemochromogens may be identical with those prepared from hemin with pyridine, the slight difference in spectra being due to experimental error or merely due to the presence in the solution of free globin. On the other hand two further suggestions may be considered. The difference may be real, the pyridine having united in the one case with alkaline heme, in the other case with hemoglobin. Data which we attempted to secure bearing on this possibility by means

<sup>1</sup> In a similar experiment in which a large excess of  $K_3Fe(CN)_6$  was used, the ratio of pigment to oxidant being 1:260, the same result was obtained. The interpretation that we were dealing with an *oxidized hemochromogen* was, however, rendered certain by the experiment (Fig. 8) in which the same pigment was obtained from MHb by the addition of pyridine.

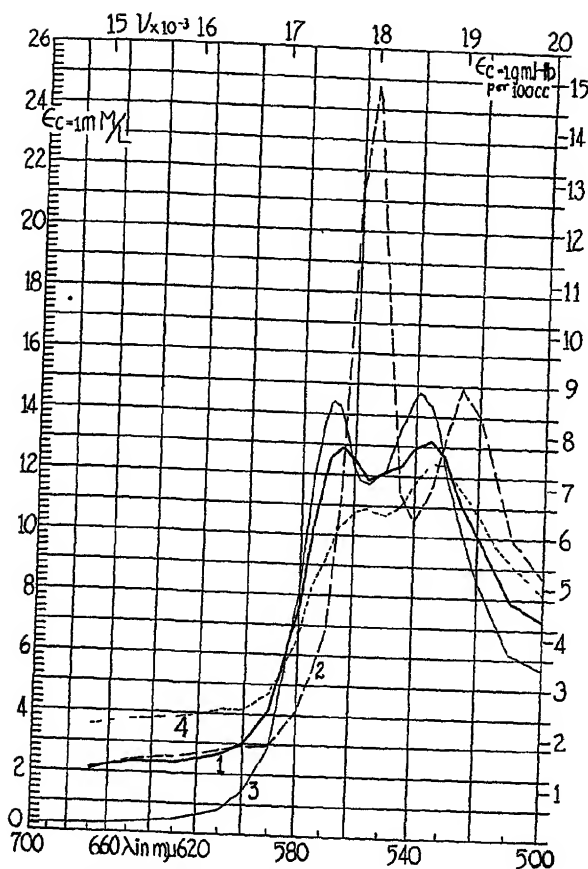


FIG. 7. Reversible change of reduced pyridine hemochromogen prepared from hemoglobin to a CO derivative. Curve 1 represents reduced pyridine hemochromogen from Hb, after treatment with CO, 0.12 mm per liter of  $\text{HbO}_2$  buffered to pH 7.0 with 0.008 M phosphate and reduced by evacuation with  $\text{N}_2$ , oxygen-free pyridine added to a concentration of 41 per cent, solution giving typical reduced pyridine hemochromogen spectrum (Fig. 6), then equilibrated with pure CO gas; Curve 2, reduced pyridine hemochromogen from previous solution. After washing out the CO with  $\text{N}_2$ , the spectrum typical of reduced pyridine hemochromogen from Hb was obtained; Curve 3,  $\text{HbCO}$ , the spectrum of  $\text{HbCO}$  from Hb for comparison; Curve 4, oxidized pyridine hemochromogen from Hb (?). Same as solution for Curve 1, but treated with illuminating gas instead of pure CO.



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of oxygen capacity determination also have proved difficult to interpret. One such group of determinations suggested that the

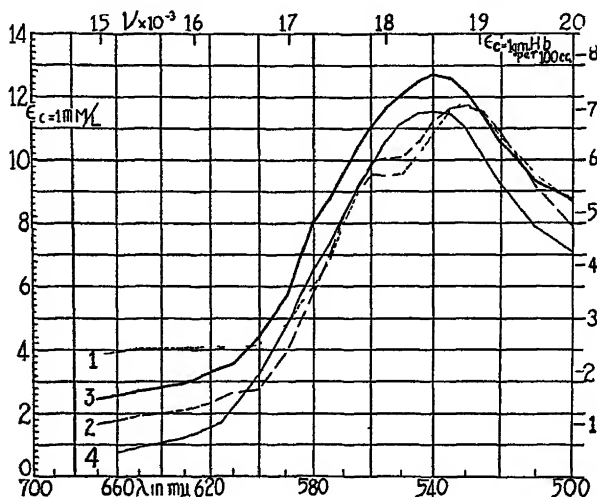
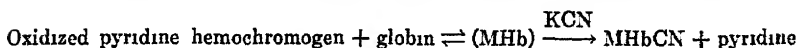
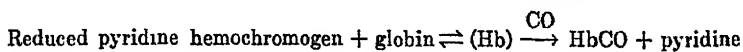


FIG 8. No alteration of spectrum of oxidized pyridine hemochromogen from Hb by addition of  $K_3Fe(CN)_6$ . Addition of KCN to oxidized pyridine hemochromogen from Hb yields cyanhemoglobin. Curve 1 represents oxidized pyridine hemochromogen from Hb (+  $K_3Fe(CN)_6$ ), 0.12 mm per liter of  $HbO_2$ , buffered to pH 7.0 with 0.013 M phosphate and reduced by evacuation with  $N_2$ , pyridine added to a concentration of 40 per cent and re-aerated, yielding a typical oxidized hemochromogen spectrum, solution then treated with 0.3 mm per liter of  $K_3Fe(CN)_6$  and read. The absorption due to ferricyanide is entirely negligible (7); Curve 2, oxidized pyridine hemochromogen from MHb, 0.11 mm per liter of  $HbO_2$ , buffered to pH 7.0 with 0.007 M phosphate, converted to MHb by addition of 3.7 mm per liter of  $K_3Fe(CN)_6$ , pyridine to a concentration of 39 per cent then added, and solution read (Upon addition of  $Na_2S_2O_4$  the solution yielded the spectrum typical of reduced hemochromogen) The absorption due to ferricyanide is negligible (7); Curve 3, MHbCN from oxidized pyridine hemochromogen (from Hb), 0.11 mm per liter of  $HbO_2$ , buffered to pH 7.0 with 0.007 M phosphate and reduced to Hb by evacuation with  $N_2$ , pyridine then added to a concentration of 37 per cent and solution re-aerated, KCN then added to a concentration of 1.4 mm per liter and solution analyzed; Curve 4, MHbCN, typical cyanhemoglobin prepared from methemoglobin for comparison

pyridine

change  $HbO_2 \longrightarrow$  oxidized hemochromogen resulted in the liberation of only one-half of the bound oxygen. The third expla-

nation is that after the addition of pyridine to hemoglobin, the solution contains a hemochromogen, consisting of pyridine combined with heme, and also undenatured globin. A new set of equilibrium conditions may favor the regeneration of hemoglobin; in the presence of CO the undenatured globin recombines with reduced heme to produce HbCO, while in the presence of KCN it recombines with oxidized heme to form cyanhemoglobin.

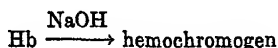


Further experiments are needed to elucidate the mechanisms involved in these phenomena. We also leave for further study the possible bearing which these observations have upon the subject of the replacement of nitrogenous bodies in hemochromogens.

#### SUMMARY

The absorption spectra of oxidized and reduced hemin and of a number of oxidized and reduced hemochromogens have been described.

Data have been presented to illustrate that the factor of time is important in the reaction



Solutions of hemoglobin treated with pyridine yield characteristic hemochromogen spectra. The reduced hemochromogen so formed reacts reversibly with CO. The oxidized form of this hemochromogen reacts with KCN, presumably yielding under our conditions cyanhemoglobin. The possible significance of these observations has been discussed.

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## SPECTROPHOTOMETRIC STUDIES

### V. A TECHNIQUE FOR THE ANALYSIS OF UNDILUTED BLOOD AND CONCENTRATED HEMOGLOBIN SOLUTIONS\*

BY DAVID L. DRABKIN AND J. HAROLD AUSTIN

*(From the Department of Physiological Chemistry and the John Herr Musser  
Department of Research Medicine, School of Medicine, University of  
Pennsylvania, Philadelphia)*

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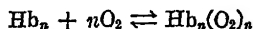
From the time spectrophotometry was first applied to the study of hemoglobin (1), the advantages of the method have been ascribed mainly to its usefulness in the study of dilute solutions. With the passing years, however, certain problems have arisen which have made desirable the application of spectrophotometry to concentrated solutions, and to blood which has not been exposed to extraneous gases after withdrawal from the body. Although no technique is more suitable than the spectrophotometric for the direct determination of mixtures of pigments (2), it has never been suggested for the determination of Hb-HbO<sub>2</sub> in blood as drawn. The dilution of blood necessitated by the use of the usual spectroscopic cells obviously has precluded such an analysis, for we are dealing here with no ordinary mixture of pigments but with an equilibrium involving also dissolved oxygen at a particular tension.

The question whether dilute hemoglobin solutions retain fully the properties of this pigment in its normal physiological environment has been considered (3) and may not be entirely academic. The high concentration of hemoglobin within the erythrocytes (30 to 35 per cent) makes pertinent an inquiry into some of the

\* Preliminary reports upon phases of this study have been presented and have appeared (*J. Biol. Chem.*, **100**, x (1933); *Am. J. Med. Sc.*, **186**, 889 (1933)).

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physical properties of hemoglobin in concentrations of this strength. The value of  $n$  in the equation



was assumed originally by Hill (4) to be determined by molecular aggregation. This explanation has remained purely hypothetical. More recently, a similar explanation has been advanced to account for the discrepant spectral behavior of certain dyestuffs—Nile blue and the simple safranines—in concentrated solution (5). The spectrophotometric study of very concentrated solutions of hemoglobin therefore appeared desirable.

The application of Beer's law to hemoglobin solutions was tested first by Butterfield (6). By means of crude cells (two plates of glass separated by a thin ring of metal and clamped together) this investigator examined spectrophotometrically moderately concentrated solutions. The highest concentration attained was that of blood diluted approximately 1:3. For the quantitative study of very concentrated solutions or whole blood unexposed to air Butterfield's type of cell is entirely inadequate. When one is dealing with diluted blood solutions the amount of material which gathers between the cover-slip and the cell wall is of no moment. However, when very thin cells are employed with viscous material this factor must be considered if reproducibility of cell depth in all determinations is to be attained. The cell furthermore should be so constructed as to admit material without contamination with environmental gases.

We have designed a spectroscopic cell<sup>1</sup> which appears admirably suited to the study of very concentrated solutions and satisfies the considerations discussed above. In the present paper this cell will be described and spectrophotometric data, hitherto unavailable, upon very concentrated solutions of hemoglobin and undiluted blood will be presented.

### *Methods*

*The Cell*—Fig. 1 represents diagrammatically a vertical section through the new cell. The drawing is to scale, except the chamber,

<sup>1</sup> Constructed for us by the Bausch and Lomb Optical Company. Cells of this type may also prove useful in the study of solutions other than hemoglobin.

*C*, which is optically ground out of the upper plate, and whose actual depth is only 0.07 mm. The light cross-hatched areas are of glass, that within the metal casing (heavy cross-hatching) being optical. The chamber of the cell is readily filled and emptied through two glass capillary tubes, connected with the interior by ground openings in a heavy glass cover, forming the floor of the chamber. (If different depths are required, several appropriately ground upper plates may be used interchangeably; the rest of the cell assembly need not be duplicated.)

*Filling and Emptying the Cell*—The cell may be filled by suction or from a small tonometer by gentle positive pressure afforded by mercury in a small reservoir connected with the tonometer. A 2

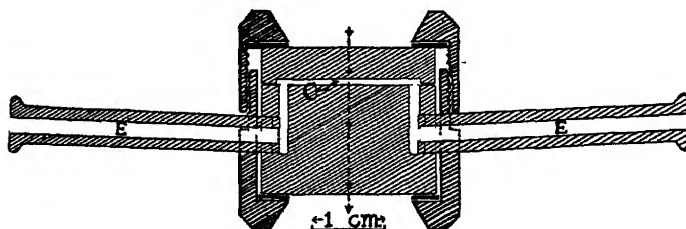


FIG 1. Vertical section through center of cell *C*, chamber of cell, depth of chamber 0.07 mm; *E*, entry and exit capillary tubes. The broken arrow indicates the direction of the passage of light through the cell. When in position for reading in the spectrophotometer, the beam of light passes horizontally through the cell.

to 2.5 cm. length of pure gum tubing with a wall 1 mm. thick and an internal bore of 3 mm. has been found suitable for holding in close approximation the entry tube of the cell and the exit tube of the tonometer. During the filling of the cell the stop-cocks of the tonometer are adjusted for a slow, gradual escape of the blood or concentrated solution. With a little practice a technique for filling the chamber without interrupting the flow of solution is easily developed. If desired, the solution under study may be flushed freely through the cell, though in most instances it is not necessary to run more fluid through than enough to overflow into the exit tube of the cell. After completion of the spectrophotometric readings the cell is washed by alternate aspiration of distilled water and air. It is then unassembled and the parts thor-

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oughly dried, a soft, well laundered linen handkerchief being used for the surfaces and a fine pipe-stem cleaner for the tubes.

*Quantity of Material Used*—In the present design of the cell the interest was in accurate determination rather than in the use of small samples. When the cell was filled by suction at least 0.5 cc. of material was needed to allow for proper immersion of the entry tube; with a tonometer as little as 0.3 cc. sufficed. It may be calculated that the volume of the chamber is only 0.021 cc., but approximately 10 times this quantity of fluid is needed for complete filling of the entry tube, the channels, and the chamber and to allow for overflow into the exit tube. For those interested in possible applications of cells of this type to work involving smaller samples, it may be suggested that merely reducing the entry and exit tubes to one-half their present length will effect a 30 per cent reduction in the total volume needed for filling.

*Calibration of Cell Depth and Balancing of Absorption Due to Cell Itself*—Although one may assume that modern methods of engine grinding can reproduce accurately specified depths such as 0.07 mm., a check upon this dimension was considered necessary.  $\text{CuSO}_4$  in ammoniacal solution, suggested by us as a standard (7), could not be made up in the desired concentration and was therefore unsuitable for the spectrophotometric calibration of the new cell. We have employed for this purpose a concentrated solution of *reduced pyridine hemochromogen* prepared from a weighed quantity of crystalline chlorohemin (8). The readings were at three wave-lengths, 560, 540, and 525  $m\mu$ , and the assumed values of the respective constants  $\epsilon$  ( $c = 1$  mm per liter) 27.4, 12.1, and 17.2, based upon measurements in a 1 cm cell (8). The calculations involve a simple transposition of terms in the equation used for deriving  $\epsilon$  ( $c = 1$  mm per liter).

$$\epsilon(c = 1 \text{ mm per liter}) = \frac{\epsilon_{\text{observed}}}{d(\text{depth})_{\text{cm}} \times c(\text{actual concentration in mm per liter})} \quad (1)$$

$$d_{\text{cm}} = \frac{\epsilon_{\text{observed}}}{\epsilon(c = 1 \text{ mm per liter}) \times c_{\text{mm per liter}}} \quad (2)$$

The concentration of the hemochromogen solution was 5.92 mm per liter. The observed  $\epsilon$  values at  $\lambda$  560, 540, and 525  $m\mu$  were respectively 1.150, 0.502, and 0.715. The substitution of these

values and the above constants in Equation (2) gave an average  $d_{\text{cm}} = 0.00704$ , and verified the depth of the cell.

The absorption due to the cell itself was "balanced out" by the use of an empty 1 cm. cell in the opposite saddle of the spectrophotometer. There are theoretical objections to this procedure, but in actual practice they were found to be of no moment and the purchase of an expensive, duplicate 0.07 mm. cell for balancing purposes was uncalled for.

*Material Studied*—Absorption measurements were carried out upon (a) dog oxalated or defibrinated blood, hemolyzed by the addition of powdered saponin to a concentration of approximately 0.2 per cent, (b) dog non-hemolyzed defibrinated blood; (c) concentrated solutions of crystallized horse hemoglobin. This material was prepared by so called isoelectric precipitation and dialysis.<sup>2</sup> Relatively saturated solutions were made up from the crystal paste by the addition of less than minimal amounts of  $\text{N NaOH}$  necessary to dissolve all the crystalline material. The supernatant solution of  $\text{NaHbO}_2\text{-HbO}_2$  was separated from the undissolved sediment by centrifuging. The pH of solutions of this type, read by the glass electrode, usually was 7.5 to 8.0. Only those solutions were employed in which the presence of "inactive" hemoglobin was presumably excluded by a comparison of the absorption constants of the pigment in the form of  $\text{HbO}_2$  and after its conversion to  $\text{MHbCN}$  and  $\text{Hb}$ .

As in our previous work, the concentration of all solutions used was determined spectrophotometrically by conversion of aliquots into  $\text{MHbCN}$  solutions of suitable strength for reading in the usual 1 cm. cell.

### Results

*Hemolyzed Dog Blood*—Absorption curves of  $\text{HbO}_2$  and  $\text{Hb}$ , which need not be presented, were obtained from five specimens of oxygenated, hemolyzed dog blood and three specimens of hemolyzed blood to which  $\text{Na}_2\text{S}_2\text{O}_4$  had been added. The concentration of pigment in the samples studied varied from 8.38 to 11.5 mm per liter.

<sup>2</sup> The data upon solutions of crystallized hemoglobin are taken from unpublished experiments of one of us (D. L. D.) and W. C. Stadie.



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The curves were virtually identical with those obtained from 1:100 and 1:1000 dilutions of blood in 1 cm. and 10 cm of cells, respectively (7, 9). There was some reason to believe that disturbing factors, such as very slight turbidity due to presence of serum, operate to a smaller extent in the new, very thin cell than in the commonly employed 1 cm. or larger cells. This was indicated by somewhat lower absorption values obtained in the blue region of the spectrum, ordinarily more affected by disturbing influences. In this connection may also be cited the mean square errors ( $m = \sqrt{\Sigma(d^2)/(n - 1)}$ ) of our determinations. The values for  $m$  compare favorably, in spite of the presence of serum, with

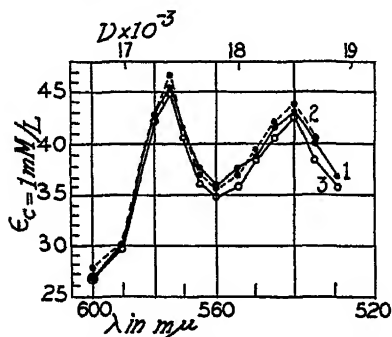


FIG 2 Absorption spectra of defibrinated, non-hemolyzed dog blood. Curve 1 represents HbO<sub>2</sub>, 10.7 mm per liter, read in a 0.07 mm cell; Curve 2, same as above, reread after standing in the cell for 30 minutes; Curve 3, data obtained after refilling of cell with a fresh sample of the same blood.

those obtained in our work with hemolyzed, washed blood cells (9), and are typified by the following: HbO<sub>2</sub> 0.08 ( $\lambda$  575  $m\mu$ ), 0.16 ( $\lambda$  560  $m\mu$ ), and 0.06 ( $\lambda$  540  $m\mu$ ); Hb 0.15 ( $\lambda$  560  $m\mu$ ), 0.28 ( $\lambda$  555  $m\mu$ ), and 0.14 ( $\lambda$  550  $m\mu$ ).

It is worthy of mention that the hemolyzed blood film in the 0.07 mm cell maintained a homogeneous character for periods of time longer than 1 hour, for similar periods of time complete reduction was maintained in Hb specimens reduced with CO<sub>2</sub> and N<sub>2</sub>, the capillary tubes of the cell being sealed only by excess blood.

*Dog Non-Hemolyzed Blood*—The absorption curves yielded by non-hemolyzed blood (HbO<sub>2</sub> concentration, 10.7 mm per liter) are depicted in Fig. 2. The extreme turbidity due to the presence of

the erythrocytes was doubtless responsible for the character of these spectra. Due to variations in the number of red cells and possibly in their size, reproducibility of absorption data from blood to blood can hardly be expected when non-hemolyzed specimens are employed. However, the following points are of interest.

1. The spectra are an expression of the effects of extreme turbidity and may prove useful in the interpretation of deviations sometimes reported in work where only slight turbidity obtains. The absorption constants at the peaks ( $\lambda$  575 and  $\lambda$  540  $m\mu$ ) are approximately 3 times the magnitude of those obtained with hemolyzed blood or solutions of hemoglobin. The whole absorption curve is flattened due to a relatively greater increase in absorption at the red and blue ends than in the mid-region of the visible spectrum. There is also evident a striking reduction in the ratios of absorption at  $\lambda$  575  $m\mu$  to  $\lambda$  560  $m\mu$ , the average ratio in the plotted results being 1.29 in comparison with the usual ratio for  $HbO_2$  which is over 1.70.

2. In spite of these physical distortions in the absorption spectrum, the curves were fairly reproducible for the blood of an individual animal (Fig. 2).

3. The blood cells showed apparently no disposition to settle towards the edge of the cell. During a 30 minute period the homogeneity of the 0.07 mm film was maintained, as judged by the absorption measurements (Fig. 2). The absence of "settling" under these conditions was an unexpected finding and is a physical phenomenon worthy of note.

*Concentrated Solutions of Crystallized Horse Hemoglobin*—Absorption data have been obtained upon six solutions of crystallized Hb, reduced by equilibration with  $CO_2$  and  $N_2$ , and upon three completely oxygenated solutions. The pigment concentration of the preparations varied from 17.3 to 25.58 mm per liter, concentrations of the order of magnitude and moderately in excess of those ordinarily found in normal erythrocytes. Since the data agreed closely, only the results upon the most concentrated solution are presented (Fig. 3). A hemoglobin concentration of 25.58 mm per liter is equivalent to 42.72 gm. per 100 cc. and represents, as far as we know, the highest concentration of the blood pigment that has been subjected to physical measurements of this type.

Fig. 3 presents the following experimental findings.

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1. The absorption curve yielded by this extremely concentrated preparation of horse HbO<sub>2</sub> is practically identical with the average curve obtained from dilute solutions of hemolyzed, washed dog erythrocytes.

2. A 25.58 mm solution is so strong that upon diluting it 1:5000 readings in a 10 cm. cell were, at the significant wave-lengths,

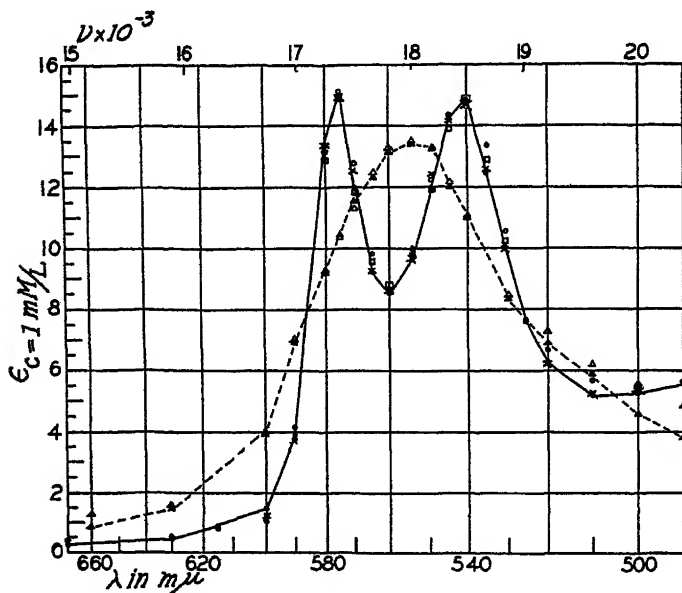


FIG 3 Absorption spectra of very concentrated solutions of crystallized hemoglobin from horse blood. ● represents HbO<sub>2</sub>, 25.58 mm per liter, read in a 0.07 mm. cell; ×, above solution diluted 1:5000, read in a 10 cm. cell; □, original solution diluted 1:10,000, read in a 10 cm. cell; ○, for comparison average of data upon HbO<sub>2</sub> from hemolyzed washed dog erythrocytes, dilute solutions read in a 1 cm. cell; ▲, Hb, 25.58 mm per liter, reduced by CO<sub>2</sub> and N<sub>2</sub>, read in a 0.07 mm. cell; △, average of data upon dilute Hb solutions from hemolyzed washed blood cells, read in a 1 cm. cell

within the range of optimal accuracy of the photometer we employ (7). Though the determinations are not quite as reliable, satisfactory readings could still be made after diluting the original solution 1:10,000. As is evident in Fig. 3, the agreement between the highly concentrated and the highly dilute solutions of HbO<sub>2</sub> is excellent.

3 The concentrated solutions in the 0.07 mm. cell yielded somewhat higher than usual ratios of extinction at  $\lambda$  575  $m\mu$  to that at  $\lambda$  560  $m\mu$ . For example, the ratio for HbO<sub>2</sub> in Fig. 3 is  $15.00/8.57 = 1.75$ , while the average ratio in our work with dilute solutions of washed blood cells in a 1 cm cell (9) was  $15.13/8.73 = 1.73$ . Such differences are perhaps not significant but they leave the impression that low ratios reported in earlier work need not be ascribed to changes in HbO<sub>2</sub> (10), but are rather the result of physical factors such as slight turbidity. As has already been said in connection with the experiments upon saponin-treated blood, such factors appear to operate only to a negligible extent in our new cell.

4. The magnitude of the absorption constants and the high value for the  $\epsilon/\epsilon$  ratio, cited above, practically exclude the presence of more than negligible amounts of "inactive" hemoglobin from this preparation of crystalline HbO<sub>2</sub>. Added proof is given in the quantitative conversion of the pigment to reduced Hb, whose absorption constants nearly duplicate the values obtained upon dilute Hb solutions (9).

#### DISCUSSION

The experiments reported in this paper have led to the following deductions.

In the quantitative investigation of hemoglobin the spectrophotometric technique offers an extraordinary latitude with respect to the concentration of materials which may be used.

Beer's law (of proportionality of concentration and absorption) has been found to hold over a very extensive range of concentrations for the pigments HbO<sub>2</sub> and Hb—an accomplishment made possible by our new cell.

The finding of strict proportionality between concentration and absorption in the case of simple compounds would practically exclude the presence of aggregation involving intramolecular rearrangement. In the case of hemoglobin we may only conclude that if molecular aggregates do exist the association complex is of such a character as to exert not the slightest influence upon the visible absorption spectrum. This must mean that in the neighborhood of the prosthetic group (Fe-porphyrin) the intramolecular arrangement is constant, though other changes in the whole

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molecule may possibly occur. It would appear that, like  $O_2$  and  $CO$ , *light is absorbed per unit of Fe-porphyrin*.

The spectral data which we have presented upon the 17.3 to 25.58 mm preparations of hemoglobin indicate, throughout the whole range of concentrations involved, that we are confronted with a behavior characteristic of true molecular phenomena. In contrast to such spectral constancy under varied conditions are the strikingly different aggregation spectra of certain metallic sols (11). The optical properties arising from the colloidal state *per se* have been thought to arise from turbidities, "optically infinitely thin" (11). We have demonstrated the character and direction of change which extraneous turbidity causes in the spectra of hemoglobin solutions. Therefore, the spectral constancy which we have found seems important in itself. We are presumably dealing with materials which, in respect to light absorption, resemble *true solutions* even in concentrations of the magnitude found in red blood cells.

### SUMMARY

A new spectroscopic cell, 0.07 mm. in depth, has been designed for the study of very concentrated solutions. It may also be employed for the study of solutions without exposure to environmental gases.

With this cell the application of spectrophotometry to the study of hemoglobin has been greatly extended. For the first time optical studies of this type have been made upon hemolyzed undiluted blood, non-hemolyzed blood, and upon solutions of crystallized hemoglobin in concentrations as high as, or in excess of, those found in red blood cells.

Beer's law has been found to apply for hemoglobin solutions in a range of concentrations from 1 to 0.0001, where 1 = 25.58 mm per liter (42.72 gm. per 100 cc.), the highest concentration studied.

The absorption of light by hemoglobin appears to be, like its union with gases, a function of the Fe-porphyrin. Therefore, aggregation involving intramolecular rearrangement within the prosthetic groups has been excluded by our measurements. No conclusions, however, as to the possibility of molecular association involving the large hemoglobin molecule could be drawn.

With respect to the specific absorption of light, hemoglobin, even in very concentrated preparations, resembles a true solution.

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# DETERMINATION OF CHLORIDES IN BIOLOGICAL FLUIDS BY THE USE OF ADSORPTION INDICATORS

## THE USE OF DICHLOROFLUORESCIN FOR THE VOLUMETRIC MICRODETERMINATION OF CHLORIDES IN CEREBRO- SPINAL FLUIDS AND BLOOD SERUM

BY ABRAHAM SAIFER AND MORRIS KORNBLUM

*(From the Division of Biochemistry of the Department of Laboratories, The  
Jewish Hospital of Brooklyn, New York)*

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Although in the past few years much work has been done on the use of adsorption indicators for the determination of halides, it has been applied mostly to pure organic and inorganic compounds (1, 2, 8). Only two references to the application of these indicators to the determination of chlorides in biological fluids were found. Osterberg (9) used an acetone extract of serum or plasma with dichlorofluorescein as the adsorption indicator. Bullock and Kirk (2), utilizing the same method, analyzed sea water and claimed to have performed analyses "on certain biological materials for a period of some years." An added note in the latter article states that "work is in progress on the application of these methods to biological fluids."

In a laboratory where many chloride determinations are performed on biological fluids, the need for a rapid, accurate micro-method is apparent. The adsorption indicator method is more accurate and rapid, requires fewer standard solutions, and is more economical than other previously described methods.

The method which has been generally used in this laboratory up to the present was a modification of the standard micro-Volhard procedure in which the precipitated silver chloride is carried down by nitrobenzene, as suggested by Caldwell and Moyer (3). The disadvantages of the Volhard method are the necessity of using more than one standard solution, of removing the precipitated



silver chloride from the solution, and the adsorption of silver ions by silver chloride (7)

Other micromethods which have been applied to the determination of chlorides in biological fluids are those of Foldes and Tauber (6) who utilized the Mohr titration method with the Folin-Wu filtrate, that of Filedt Kok (5) who precipitated the proteins with sulfosalicylic acid and titrated an aliquot of the filtrate with mercuric nitrate in the presence of sodium nitroprusside; and that of Fairhall and Heim (4) who use an iodometric procedure.

The authors attempted to apply the method of Osterberg to both macro- and microdetermination of chlorides in blood serum. The method consists of adding 2 ml. of serum or plasma to 7 ml. of acetone in a centrifuge tube, making up to 10 ml. with acetone, shaking, and centrifuging. A 5 ml. aliquot is titrated with N/35.46 silver nitrate. According to the author, a 5 ml. aliquot represents one-half the chloride content of the sample. However, the volume of the precipitate is not taken into consideration. Even when centrifuged at high speeds (2200 R.P.M.), it amounts to 0.7 ml. out of a total volume of 10 ml. Since we have found that the residual precipitate contains practically no chlorides, the latter are then all dissolved in an actual volume of about 9.3 ml., and it must follow that the values obtained by Osterberg are high.

When the Osterberg method was applied to microdetermination by adding 0.2 ml. of serum to 1.8 ml. of acetone, lower results were obtained as compared to the original macromethod. This is probably due to the low solubility of chlorides in acetone. A solvent was then sought which would completely precipitate the proteins (that interfere with the titration), which would give an end-point as sharp as that obtained with acetone, and which has a higher solubility for chlorides than acetone. An alcohol-ether solution (3:1) was found to give excellent results.

The method finally adopted was that of adding a known amount of serum to a given volume of alcohol-ether, shaking thoroughly, centrifuging, and then titrating the clear supernatant fluid, in the same tube, with standardized silver nitrate, dichlorofluorescein being used as the indicator. For cerebrospinal fluids, a measured amount was diluted with a small volume of water, approximately 3 times the volume of alcohol-ether was added, and the solution titrated directly with standardized silver nitrate in the presence of the indicator.

## EXPERIMENTAL

*Solutions*

Standard sodium chloride. Solution A. 10,000 gm of dry c.p. NaCl in 500 ml. of solution; 1 ml. equivalent to 20 mg. of NaCl. Solution B. 100 ml of Solution A diluted to 1000 ml ; 1 ml. equivalent to 2.00 mg of NaCl. Solution C. 0.500 gm of NaCl in 1000 ml. of solution; 1 ml. equivalent to 0.500 mg. of NaCl.

TABLE I  
*Determination of Chlorides in Cerebrospinal Fluid (0.20 Ml Samples)*

	NaCl found	Average NaCl found	Added NaCl recovered
	mg	mg	mg
Sample 1	1.625	1.622	
	1.625		
	1.613		
	1.625		
0.500 mg. NaCl added, Solution C	2.120		0.498
	2.109		0.487
	2.120		0.498
	2.120		0.498
Sample 2	1.349	1.356	
Cerebrospinal meningitis	1.360		
	1.360		
	1.349		
	1.360		
0.500 mg. NaCl added, Solution C	1.856		0.500
	1.868		0.512
	1.868		0.512
	1.856		0.500
	1.856		0.500

Standard silver nitrate. An approximately 0.02 N solution of AgNO<sub>3</sub> was made. The standardization was performed against the standard NaCl Solution B by the following procedure. The sample, contained in a test-tube of convenient size (6 ×  $\frac{3}{4}$  inches) was titrated with the silver nitrate, with a 5 ml. microburette calibrated in 0.02 ml. divisions. To 2 ml. samples of standard sodium chloride Solution B (containing 4.00 mg. of sodium chloride) were added 3 ml. of alcohol-ether and 1 drop of the indi-

cator. The samples were then titrated with silver nitrate (approximately 0.02 N) until the first definite pink color appeared throughout the solution. The standardizations were performed with sodium chloride Solutions B and C with excellent results.

TABLE II

*Effect of Varying Amounts of Alcohol-Ether, Blood Serum (0.20 Ml. Samples)*

Alcohol-ether	NaCl found	Average NaCl in serum	Added NaCl recovered
<i>ml</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>
1 00	0 945	1 072	
3 00	0 965		
5 00	1 072		
7 00	1.072		
10 00	1 072		
0 500 mg. NaCl added, Solution C			
1 00	1 418		0 346
3 00	1 567		0 495
5 00	1 556		0 484
7 00	1 556		0 484
10 00	1 578		0 506

TABLE III

*Determination of Chlorides in Blood Serum (0.2 Ml. Samples)*

	NaCl found	Average NaCl in serum	Added NaCl recovered
	mg	mg	mg
Sample 1	1 129	1 138	
	1 140		
	1 140		
	1 140		
	1 140		
0 500 mg. NaCl added, Solution C	1 648		0 510
	1 648		0 510
	1 648		0 510
	1 636		0 498
	1.648		0 510

Alcohol-ether A 3:1 solution by volume was made by mixing the appropriate quantities of 95 per cent ethyl alcohol with U.S.P. ethyl ether

Dichlorofluorescein indicator. Eastman Kodak Company's No. 373, 0.05 per cent in 70 per cent alcohol.

*Cerebrospinal Fluids*—0.2 ml. of fluid was pipetted into a test-tube of 30 ml. capacity, the sides of the tube were washed with 1 cc. of distilled water, and 3 ml. of alcohol-ether and 2 drops of the indicator were added. The solution was titrated with silver nitrate solution to the first faint pink color. The proteins were not precipitated under these conditions. The end-point was sharp. The results of a series of tests on cerebrospinal fluids, including recoveries of added sodium chloride, are shown in Table I.

*Blood Serum*—0.2 ml. of serum was added, with shaking, to 5 ml. of alcohol-ether in a 15 ml. centrifuge tube. The tube was stoppered, shaken thoroughly, and centrifuged at high speeds (above 2000 R.P.M.). 2 drops of indicator were added and the clear supernatant fluid was then titrated in the same tube with the standardized silver nitrate until the first definite pink color appeared throughout the solution.

Table II illustrates the effect of using various amounts of alcohol-ether. Table III shows the results of a series of blood serum determinations including recoveries of added NaCl.

#### DISCUSSION

The results obtained in the above experiments indicate the high accuracy and reproducibility of the adsorption indicator method for the determination of chlorides in spinal fluid and serum. Quantities in the range of 1 mg. of sodium chloride in spinal fluid with a maximum deviation<sup>1</sup> of 1.0 per cent and in blood serum with a maximum deviation of 2 per cent can be determined by this method. Increased accuracy may be obtained by the use of more dilute silver nitrate but with a consequent loss in the sharpness of the end-points.<sup>2</sup> Another advantage of this method is that the complete analysis can be performed in a single tube without transfer.

<sup>1</sup> These deviations, it will be observed, involve a maximum quantity of 0.02 ml. of AgNO<sub>3</sub>. In the great majority of cases, the deviation is 0.01 ml. It is expected that the use of burettes having finer calibrations should lower the percentage deviation considerably.

<sup>2</sup> It has been found that the addition of small amounts of caprylic alcohol sometimes increases the sharpness of the end-point.

That the results obtained by this method are in close agreement with those obtained by the Volhard method is shown by the fact that the average of four runs for spinal fluids gave a value of 731 mg per cent, while the adsorption indicator method gave an average value of 735 mg per cent. For serum, with the Wilson and Ball (10) method, the average of five runs was 575 mg. per cent, whereas that obtained by the adsorption indicator method, on the same serum, averaged 563 mg per cent for the same number of determinations. In general, the Wilson and Ball procedure gave higher results than the adsorption indicator method.

Further work is now in progress on the application of adsorption indicators to the determination of chlorides in other biological fluids, *e g.* urine, pleural fluid, etc.

#### SUMMARY

A rapid, accurate method for the microdetermination of chlorides in spinal fluids and in alcohol-ether extracts of serum has been described, in which dichlorofluorescein is used as an adsorption indicator and standard silver nitrate is used for titration. The entire procedure is carried out in a single tube without transfer. The method is accurate within the limits of error of the apparatus used.

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## A CHEMICAL HYGROMETER

BY THORNE M. CARPENTER

*(From the Nutrition Laboratory, Carnegie Institution of Washington, Boston)*

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The determination of the amount of water vapor in atmospheric air or gaseous mixtures has usually been made by gravimetric methods or by psychrometers. In the gravimetric method a measured volume of air is passed through containers of absorbents that can be weighed before and after the air has passed. The increase in weight gives the amount of water vapor in the volume of sample. Both of the above methods have their disadvantages. The gravimetric method takes considerable time and requires absorption units, meters, and balances. The psychrometric method is unreliable as it depends so much on rapidity of wind movement and freshness of the covering of the wet bulb. A third method which has been little used is that of determining by volume the water vapor in the air as one would determine the amount of  $\text{CO}_2$ ,  $\text{O}_2$ , and other gases. For this method two types of apparatus may be used, one in which the unknown gas is dried by means of an absorbent, and the other in which the gas is saturated with water vapor and from the known volume increase, which would occur with a dry gas when saturated at the temperature of the determination, a calculation is made of the original volume of water vapor in the same. The former principle has been used by Schwackhofer (1), Edelmann (2), Pettersson (3), Rideal and Hannah (4), and Mayo and Tyndall (5). The principle of complete saturation has been employed by Matern (6) and more exactly by Söndén (7).

The apparatus to be described has been devised in connection with the general program of the Nutrition Laboratory on the study of heat regulation, paths of heat elimination, and water balance of humans and animals. Although it was designed primarily to measure by volume the water vapor in the air currents

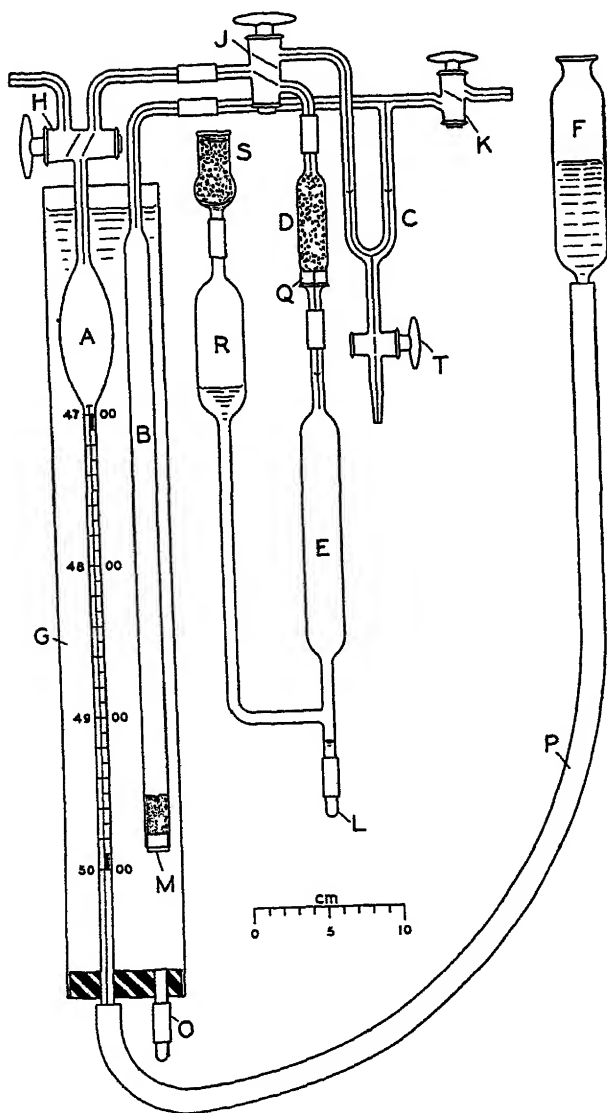


FIG. 1. Diagram of the chemical hygrometer *A*, measuring burette; *B*, compensator; *C*, manometer with oil; *D*, absorption pipette containing dehydrite, *E*, seal for pipette *D*; *F*, leveling bulb; *G*, water jacket; *H*, stop-

of respiration apparatus, particularly of the open circuit type, it can be applied for the determination of water vapor content generally of atmospheric air or of gaseous mixtures. The apparatus is portable and easily reproducible and has at the same time sufficient accuracy for the purpose desired. Several of these apparatus have been constructed and a comparison among them shows that, on the average, well agreeing results can be obtained.

### *Chemical Hygrometer<sup>1</sup>*

The general design, structural details, sizes of burette and capillaries, and manipulative technique have been arrived at through a number of years of experience with various forms of gas analysis apparatus devised in this laboratory (8).

*Principle*—The general principle on which the apparatus is arranged is that in which the gas is measured in a burette and the water vapor is then removed by a suitable absorbing reagent and the gas again measured. The difference represents the volume of water vapor. The volumes in the burette are counterbalanced against a gas confined in a compensator and dried by phosphorus pentoxide. A U-shaped manometer containing oil is the basis of adjustment of equilibrium

The apparatus is illustrated diagrammatically in Fig. 1. It consists of a measuring burette, *A*, a compensator, *B*, a manometer, *C*, an absorption pipette, *D*, a pipette, *E*, for an air seal with reservoir *R*, a mercury leveling bulb, *F*, a water jacket, *G*, and four stop-cocks, *H*, *J*, *K*, and *T*.

*Burette*—The burette is of 50 cc. total capacity and is graduated for 3 cc. so that each 1.0 mm. in length is equivalent to 0.01 cc.; i. e., 0.02 per cent of the total volume. The burette has at the top the 3-way stop-cock *H* and at the bottom is connected with the

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<sup>1</sup>The glass parts were made by Macalaster, Bicknell and Company, Cambridge, Massachusetts.

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cock of burette, *J*, stop-cock connecting manometer or pipette to burette; *K*, stop-cock for opening compensator to outside air; *L*, seal for pipette *E*; *M*, rubber stopper to close compensator *B*; *O*, tube for draining water jacket; *P*, nitrometer tubing; *Q*, glass stopper for closing pipette *D*, *R*, reservoir for pipette *E*; *S*, calcium chloride tube for protection of sulfuric acid in *E*, *T*, stop-cock for manometer *C*.



heavy walled rubber tubing, *P*, which in turn is connected with the leveling bulb, *F*. The total length of the burette is 66 cm. from the top of the two capillaries above the burette to the point at which the junction is made with rubber tubing.

*Compensator*—The compensator, *B*, is a cylindrical tube 15 mm in outside diameter and 40 cm in length and is connected with a capillary tube at the upper part. It is open at the lower end. When it is inverted about 3 gm. of phosphorus pentoxide are put into the compensator. A tight fitting rubber stopper is then put in at the point *M* and is sealed with paraffin. This arrangement makes it possible to renew the phosphorus pentoxide whenever it appears to have absorbed moisture and to have become liquid.

*Manometer*—The manometer, *C*, is of capillary tubing of about 2.5 mm. internal diameter; at the bottom is joined a 1-way stop-cock, *T*. The manometer, *C*, is partly filled with a non-volatile paraffin oil. This oil apparently gives off no vapor and consequently is suitable to act as a manometer liquid in this apparatus.

*Absorption Pipette*—The absorption pipette, *D*, is a cylinder of 20 mm. outside diameter and 75 mm in length. It is closed at the bottom with a solid tight fitting glass stopper, *Q*, through which a capillary hole is bored and to which is fused a piece of capillary tubing. This pipette is filled with approximately 10 gm. of dehydrite (magnesium perchlorate-trihydrate,  $\text{Mg}(\text{ClO}_4)_2 \cdot 3\text{H}_2\text{O}$ ) (9) and then the glass stopper, *Q*, is inserted and sealed with stop-cock grease of a high melting point.

*Air Seal*—In order to seal off the lower end of the absorption pipette, *D*, from the air, the pipette, *E*, is attached, holding approximately 70 cc of crude sulfuric acid. This is sealed off at the bottom at *L* with a piece of rubber tubing and a screw pinch-cock and approximately 1 cc. of mercury.

*Water Jacket*—The water jacket, *G*, is a straight cylindrical glass tube of 7.5 cm. outside diameter, into the bottom of which is fitted a rubber stopper. A tube, *O*, is inserted into the rubber stopper for draining the water from the water jacket.

*Stop-Cocks*—Stop-cock *J* is a 3-way stop-cock which permits the connection of the burette, *A*, to the absorption pipette, *D*, or to the oil manometer, *C*. Stop-cock *K* is an oblique 1-way stop-cock which permits opening of the air in the compensator, *B*, to the outside atmosphere for adjusting the level of the oil in *C*. Stop-

cock *H* is part of the burette and stop-cock *T* is for the purpose of filling the manometer, *C*

### *Manipulation*

All parts of the apparatus must be cleaned and dried. The burette should be calibrated, as is done with all other gas analysis apparatus. All parts of the apparatus are connected with pure gum black rubber tubing of suitable size and the rubber connections are coated with an acid-proof varnish<sup>2</sup> to prevent diffusion of water through the rubber. The mercury leveling bulb, *F*, is connected with the burette by means of nitrometer tubing. The manometer, *C*, is filled with a non-volatile paraffin oil of low viscosity. The absorption pipette, *D*, is filled with dehydrite and the air seal, *E*, with crude sulfuric acid in such a quantity that, when at atmospheric pressure, it will come to a level at the mark on the capillary at the upper end of the pipette. It is essential that the reservoir, *R*, of the pipette, *E*, which permits the rise and fall of the sulfuric acid, shall be so low that when the pipette, *E*, is filled with sulfuric acid, the level of liquid in the reservoir will be up in the widest part of the reservoir. This avoids drainage which may take place if so small a quantity of acid is used that when the levels are set the acid comes to a point in the narrow part of the tubing. When continual drainage takes place, it requires continual resetting of the level in the sulfuric acid pipette. The mercury should be clean and dry

With the apparatus completely assembled, the manometer, *C*, is adjusted to the open air, with stop-cock *K* open and the plug of *J* removed. Stop-cock *K* may then be closed and the plug of *J* put back. Stop-cock *H* is turned so that connection is made between the burette, *A*, and the absorber, *D*. The apparatus is now ready for preliminary sweeping to remove all traces of water vapor in the apparatus. This is done by raising and lowering the mercury bulb, *F*, ten to fifteen times with stop-cock *J* turned in such a way that there is a connection between the burette, *A*, and the pipette, *D*. The sulfuric acid is set at the mark in *E* and when this is done it is essential that the pressure in the burette, *A*, and in *E* should be at practically the atmospheric pressure. If this is not

<sup>2</sup> A special varnish sold by Arthur H. Thomas and Company, Philadelphia, for coating the wooden parts of gas analysis apparatus

the case when the sulfuric acid level is set, the quantity of sulfuric acid is altered so that the air above *E* will be at nearly atmospheric pressure when the acid comes to the mark in the narrow tube above *E*. Stop-cock *J* is now turned so that connection is made between the burette, *A*, and the oil manometer, *C*, and the level of the oil is adjusted so that it is at the same height on both sides of the U-tube. The reading in the burette is now made. Stop-cock *J* is then turned so that connection is made between the burette, *A*, and the pipette, *D*, and the mercury leveling bulb, *F*, is raised and lowered five times. Adjustments are made as before for a reading, and if all water vapor had previously been removed and the apparatus is tight, the readings should agree within 0.002 cc. The apparatus is now ready for analysis of air containing water vapor. Stop-cock *H* is now turned to the open air or to the connection with the sample. The sample is drawn in by washing the burette three times completely with the air to be analyzed, and then drawing in air until the burette is full, and adjusting it to outside atmospheric pressure at first, and subsequently to the oil manometer, *C*. Under this condition the reading is made of the volume in the burette. The adjustment can be made so quickly with this apparatus that we have found it not necessary to make a duplicate reading of the volume. The gas is then passed through the pipette, *D*, into *E* fifteen times and its volume is read on the burette, *A*. The gas is again passed through the absorbent five times and a second reading is made. These readings should agree within 0.002 cc. The difference between the last reading, corrected for the burette correction, and the first reading, corrected for the burette correction, divided by the latter, gives the percentage of water vapor by volume in the sample of air analyzed.

The essential precaution is to have the temperature of the water in the water jacket, *G*, at least 1° higher than the room temperature and the temperature of the air at which the sample was drawn. This is to avoid condensation of moisture in the burette. If condensation takes place, it is obvious that an incorrect measurement of the volume of water vapor is made. With this apparatus it is therefore unnecessary to calculate either relative humidity or partial pressure of water vapor; if desired both of these can be calculated provided the temperature of the original sample is known, especially in case of calculation of the percentage humid-

ity The results of percentage by volume can then be applied directly to the measured total volume of air coming from an open circuit apparatus and then the product reduced to 0° and 760 mm. By the factor of conversion of liters of water vapor to gm. of water vapor, one can then obtain the weight of water in the air current which has passed through the apparatus.

*Precautions in Use of Apparatus*—It is absolutely necessary that the apparatus be dried and that no moisture of any kind be used in making connections of the apparatus with samplers. The frequently used procedure of moistening a piece of rubber tubing must be avoided. The quantity of dehydrite used has been arbitrarily set as sufficient for 100 analyses. It should then be renewed. Experience thus far has not taught us as to how long the sulfuric acid is sufficiently active to protect the dehydrite in pipette *D*, but we have thus far found no evidence that it will not suffice for the 100 analyses prescribed for the dehydrite. When the apparatus is not being used, the sulfuric acid container should be protected from the open air by a calcium chloride tube, *S*, and the calcium chloride should be renewed as soon as there is any appearance of moisture on the part nearest the open air. Similarly, after the analyses are over, stop-cock *K* is opened to the outside air, and should be protected with a calcium chloride tube, as otherwise the phosphorus pentoxide in *B* will take up moisture and will soon become liquid. When the apparatus is not in use, stop-cocks *H* and *J* should be closed.

#### *Standardization of Apparatus*

The chemical hygrometer was checked by comparing the results obtained with the percentage of water vapor by volume secured from simultaneous absorption of water from an air current by means of sulfuric acid. The air was passed at the rate of 30 to 40 liters per minute through a pair of weighed Williams bottles containing between 250 and 550 cc. of sulfuric acid, and then to a calibrated dry gas meter, and then into the open air. The air was passed a definite period of time, readings being taken of the temperature of the meter at the beginning and end, and of the volumes recorded by the meter at the beginning and end. An observation of the barometric pressure was made sometime during the period of measurement. During the time of the absorption by sulfuric

acid, samples were periodically taken directly into the chemical hygrometer from the air current for analysis at a point before it entered the sulfuric acid and analyzed as rapidly as possible. An example of the comparison of results obtained by the absorption method and by the gas analysis method is given in Table I. In

TABLE I

*Example of Comparison of Determination of Water Vapor by Chemical Hygrometer with Absorption by Sulfuric Acid from an Air Current*  
January 10, 1935.

<i>Absorption by Sulfuric Acid</i>	
Duration of period = 37 min	
Total ventilation through meter = 1099.7 liters	
Average temperature of meter = 24.0°	
Barometric pressure = 752 mm	
Gains in weight of sulfuric acid containers. No 3, 7 16 gm.; No 11, 0 07 gm.	
Calculation of percentage water vapor by volume	
Total ventilation of meter	1099 7    liters
Reduction to 0° and 760 mm.	0 909
Meter factor .	0 968
Total ventilation reduced	967 64    liters
Volume of water vapor absorbed ( $7\ 23 \times 1\ 244^*$ ) =	8 99    "
Total air passed through system	= 976 63    "
Volume water vapor	= 8 99    "
Total volume	976 63    "
= 0 921% water vapor by volume	

*Results with Chemical Hygrometer*

Apparatus 12 per cent	Apparatus 0 per cent
0 938	0 938
0 906	0 907
0 908	0 921
0 917	0 922

\* Factor for conversion of gm of water to liters of water vapor.

the absorption method both the sulfuric acid containers absorbed water vapor from the air current. The total volume of ventilation during 37 minutes was 1099.7 liters, as observed, the temperature was 24.0°, and the barometric pressure 752 mm. The total volume of ventilation through the meter was reduced to 0° and 760 mm. by the factor corresponding to the temperature and the

barometric pressure, and the factor correction for the meter which had been previously calibrated. Since the air had been dried before passing through the gas meter, no correction was necessary for water vapor. This gave 967.64 liters. Obviously, to obtain the total volume of air that entered the sulfuric acid containers and from which the water vapor was absorbed, it is necessary to add the volume of water vapor to the total volume of air passing through the meter. This is obtained by multiplying the weight of water, 7.23 gm., by the factor 1.244, that is the conversion factor of weight of water to liters of water vapor. This gives 8.99 liters, thus making a total of 976.63 liters having passed through the ventilation system. To obtain the percentage of water vapor by volume in this amount of air, the volume of water vapor obtained from the above calculations, 8.99 liters, was divided by the total volume of air that entered the sulfuric acid containers, namely 976.63 liters. This gives 0.921 per cent water vapor by volume. In this particular case, two chemical hygrometers were used and three analyses were made from samples taken during the course of the ventilation. The first pair of samples was taken immediately at the beginning of the ventilation through the system and the last pair at the exact end of the ventilation through the system. The other pair was taken practically midway between the beginning and end of the period. The two chemical hygrometers gave on the average 0.917 per cent and 0.922 per cent water vapor by volume. A large number of comparisons of this character were made in order to establish the reliability of the method.

A series of 57 such comparisons with a range of 0.28 to 2.20 per cent and an average of 1.17 per cent gave an average difference of  $\pm 0.013$  per cent and a standard deviation of the differences of 0.0172 per cent. A series of thirty-three duplicate pairs from samples, the content of water vapor of which averaged 1.74 per cent, gave an average difference of 0.011 per cent and a standard deviation of the differences of pairs of 0.0143 per cent. A series of 133 pairs of analyses with an average content of 0.84 per cent made on two different apparatus gave an average of the differences of 0.011 per cent and a standard deviation of the differences of 0.0144 per cent.

*Application of Chemical Hygrometer*

The chemical hygrometer can be used as a standardizing instrument for other types of apparatus for the determination of the water vapor content of air. In this laboratory it has been used as a control instrument for the electrical psychrometer of Noyons (10). This psychrometer is composed of two exceedingly sensitive multiple thermocouples, one of which serves as a wet bulb. A series of measurements was carried out with this apparatus in which the *voltage* at 25° for a given amount of water vapor in the air was measured and simultaneous determination of the volume of water vapor was made by the chemical hygrometer. A series of such comparisons gave a calibration curve for the Noyons electrical psychrometer. Subsequently the psychrometer was installed in the laboratory for investigation on animal nutrition at the University of New Hampshire. A series of eighteen comparisons on 9 days gave an average of 1.33 per cent water vapor by the electrical psychrometer and 1.31 per cent by the chemical hygrometer. The average of the differences between pairs of comparisons was  $\pm 0.02$  per cent. The psychrometer of Noyons, as calibrated by this method, is of great advantage in securing instantaneous readings and thus rapid fluctuations are easily followed.

## SUMMARY

An apparatus is described for the determination of water vapor by volume in air. The apparatus is constructed on the principle of a gas analysis apparatus in which the volumes before and after removal of water vapor by an absorbent are read against a constant volume kept dry by phosphorus pentoxide. A large number of comparisons with results obtained by weighing the water vapor absorbed from air currents containing 0.3 to 2.2 per cent show an agreement of under 0.02 per cent on the average. The apparatus is transportable and commercially easily reproduced.

I wish to acknowledge the assistance of Mr. George Lee and Miss Elsie MacLachlan in making the gas analyses, of Mr. Basil James in making the standardization with the absolute method, and of Mr. V. Coropatchinsky in the application of the electrical psychrometer.

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## ON THE QUANTITATIVE MEASUREMENT OF THE ENZYME TYROSINASE

BY MARK GRAUBARD AND J. M. NELSON

*(From the Department of Chemistry, Columbia University, New York)*

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In connection with securing highly active tyrosinase preparations it was found necessary to know the relative enzyme content of different preparations, as well as the relative activities of different preparations, from the standpoint of unit dry weight. To obtain quantitative information of this sort, obviously a quantitative unit of measurement is necessary. The necessity for such a unit is further emphasized in following the yields of the enzyme in various purification processes, and in selecting different sources for the enzyme. In looking over the literature, it was found that Richter in 1934 (6) and others have attempted quantitative studies of the activity of tyrosinase. However, due to the nature of the study of the enzyme as it is being carried on in this laboratory, it has been found convenient to adopt a new unit for measuring tyrosinase. The magnitude of the unit selected is the amount of enzyme, which, under conditions given below, will bring about 10 c.mm. of oxygen uptake per 1 minute. In the experiments the rate of oxygen uptake was measured by means of a Barcroft-Warburg respirometer, with flat bottomed 50 cc. reaction flasks, the apparatus being run at about 90 complete oscillations per minute. The temperature was 25°. The reaction mixtures consisted of 4 cc. of 0.1 per cent *p*-cresol, a desired volume of the enzyme solution, 2 cc. of phosphate (0.2 M)-citrate (0.1 M) buffer (pH 6.2 for the reaction mixture), and sufficient water to make the final volume in the reaction flask equal to 8 cc. The pH value 6.2 was selected because it is near the optimum hydrogen ion concentration for tyrosinase action, and also because the addition of the substrate or enzyme solutions causes no appreciable change in pH of the mixture as is usually the case at other hydrogen ion concentrations.

By plotting the oxygen uptake in c.mm. against time in minutes, and comparing the slopes, corresponding to the earlier part of the reaction, it was found that, when low enough concentrations of the enzyme were employed, the slopes were directly proportional to the amounts of enzyme used, and hence could serve as a quantitative measure of the relative amount of enzyme present. When too large quantities of the enzyme were used (2 cc. of enzyme solution, see Fig. 1, A), it was found that the enzyme was less efficient, and hence the direct proportionality between the rate of

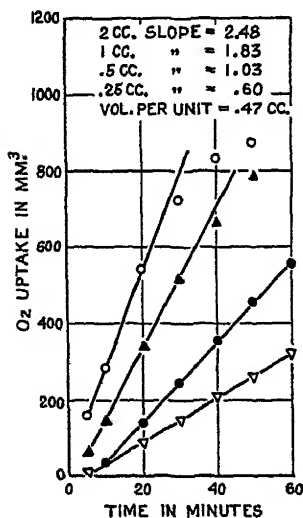


FIG. 1, A

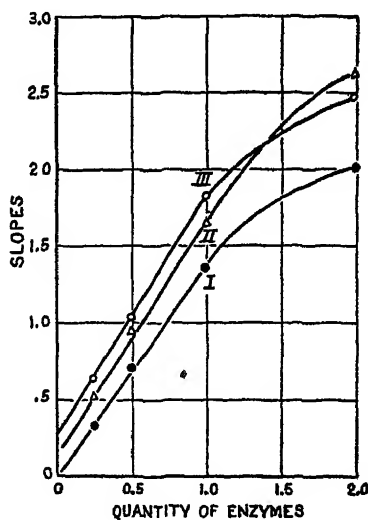


FIG. 1, B

FIG 1. Alumina-dialyzed Preparation E-III

oxygen uptake and quantity of enzyme used no longer held. Judging from the curves in Fig. 1, A, it will be seen that the addition of 0.47 cc. of alumina-dialyzed Preparation E-III, diluted 2 times, to the reaction mixture would make the time required for 10 c.mm. of oxygen uptake 1 minute. In other words, 0.47 cc. of this diluted preparation of tyrosinase contained 1 unit of the enzyme, and hence 235 cc., for example, of the undiluted preparation would contain  $235/0.24 = 980$  units (last value in Table I).

It is often desirable to know the degree of purity or activity, as some prefer to regard it, of an enzyme preparation in the dry or

solid state. This obviously can be ascertained by evaporating a given volume, containing a known number of units of the enzyme, to dryness, and then expressing the degree of purity either as dry weight per unit of enzyme activity or the number of units in a selected weight of the enzyme material. The weights given in Table I were obtained by evaporating 16.13 cc. of the enzyme

TABLE I

*Data Showing Number of Tyrosinase Units Contained in Given Volume of Juice from Given Weight of Mushrooms, and Influence of Certain Purification Processes on Activity per Unit Weight of Solid Matter*

3 pounds of mushrooms were divided into three equal lots, each weighing 435 gm. Each lot was pressed in a hydraulic press, and 235 cc of juice were obtained in each case

	Preparation No	Weight of dry solids in 16.13 cc	Cc containing 1 unit		Total units in 235 cc	Dry weight per unit
			Diluted 1 5	Undiluted		
	(1)	(2)	(3)	(4)	(5)	(6)
Plain juice	E-I E-II E-III	<i>gm</i>				<i>mg</i>
		0.8020	0.85	0.17	1385	8.4
		0.7835	0.82	0.16	1468	7.7
		0.7565	0.50	0.10	2350	4.7
			Diluted 1 3			
Alcohol-dialyzed	E-I E-II E-III	0.0215	0.85	0.28	840	0.37
		0.0230	0.64	0.21	1119	0.30
		0.0170	0.50	0.17	1382	0.18
			Diluted 1 2			
Alumina-dialyzed	E-I E-II E-III	0.0045	0.73	0.36	653	0.10
		0.0075	0.58	0.29	810	0.13
		0.0080	0.47	0.24	980	0.12

solutions according to the method devised by Dr. Saul, of this laboratory, for determining the time value of invertase. A description of Dr. Saul's procedure is given by Lutz and Nelson (2).

The enzyme preparations used in the present study were obtained from common mushrooms (common or field mushroom, *Psalliota campestris*). The mushrooms were broken up and

placed in a press and the juice collected and filtered. Such a preparation is referred to as *plain juice*.

Part of the plain juice was set aside for experimentation and the remainder cooled and rapidly mixed with 2 volumes of 95 per cent alcohol precooled to  $-10^{\circ}$ . The precipitate formed was filtered as rapidly as possible and then taken up in water equal to the original volume of enzyme. This solution of the alcohol-precipitated enzyme was next dialyzed in a rocking dialysis apparatus (for description see Lutz and Nelson (2)) for 3 days against cold running water. This preparation will be referred to as the *alcohol-dialyzed* enzyme solution.

Part of the alcohol-dialyzed preparation was set aside for experimentation and the rest was adsorbed on a suspension of aluminum hydroxide at pH 6.0, at which pH the tyrosinase is adsorbed. The suspension was then filtered off and the enzyme eluted at pH 8.0 by means of secondary sodium phosphate, and the solution containing the enzyme dialyzed in the same way as in the case of the solution of the alcohol precipitate, described in the preceding paragraph, and after dialysis the resulting solution was made up with water to equal the volume of the original enzyme solution. This solution of the enzyme will be designated as the *alumina-dialyzed* preparation.

Fig. 1, A contains an example showing how the number of cc. of a given preparation of tyrosinase, containing 1 unit of the enzyme, was determined. It will be observed that, within the experimental error, the slopes of the curves (c.mm. of oxygen uptake against minutes) for the earlier stages of the reaction are practically proportional to the amount of preparation used in the reaction mixture. This direct proportionality is emphasized more clearly in Fig. 1, B, in which the slopes for three series of rate curves (each series similar to the series shown in Fig. 1, A), corresponding to three different preparations of tyrosinase, have been plotted against the respective amounts of enzyme preparation. Up to 1 cc. of enzyme preparation, each series giving a straight line, which shows that the rate of oxygen uptake, increases directly with the amount of enzyme preparation used. When, however, too high concentrations of enzyme were used (more than 1 cc. of the preparations), then it will be noticed that the curves in Fig. 1, B start to bend towards the axis, representing the amount of

enzyme used, showing that at these higher concentrations of enzyme the latter is not acting catalytically at its maximum efficiency

Column 5 in Table I shows that in this particular process of purification of the enzyme about one-third of the total enzyme originally present in the plain juice was lost in the following steps: precipitation with 2 volumes of alcohol, redissolving in water, dialysis against running water, and finally making the dialyzed solution up to the volume of the original juice, 235 cc. Adsorbing the enzyme from the dialyzed alcohol-precipitated material to alumina, eluting, and again dialyzing and making the final volume up to 235 cc. was accompanied by an additional loss of 25 per cent of the enzyme. On the basis of dry weight per unit of enzyme, the alcohol treatment yielded a preparation 23 times more active than the solid matter contained in the original juice, and the alumina treatment produced a preparation 3 times more active than the solid matter obtained by the alcohol treatment.

Certain investigators in this field (Onslow and Robinson (3), Richter (6), and others) hold that the enzyme which catalyzes the oxidation of *p*-cresol is different from the one which brings about the oxidation of the dihydroxyphenol, catechol. In our previous paper (1) results were obtained when *p*-cresol and catechol were subjected to the action of potato tyrosinase, which pointed strongly towards the view that the same enzyme catalyzed the oxidation of both of these phenols, as suggested by Raper (5) and Pugh (4). To follow up this question still further a comparison has been made of the activities of preparations of tyrosinase from mushrooms on both these substrates from a quantitative standpoint. For the purpose the same enzyme preparations (Nos. E-I, E-II, and E-III) were used as in the *p*-cresol experiments, and indicated in Table I. Also the same reaction mixtures were used, except 4 cc. of 0.1 per cent catechol instead of the *p*-cresol. As pointed out in the previous paper, the oxygen uptake is more rapid in the case of catechol, and soon reaches a maximum, after which the reaction stops. For this reason, instead of determining the number of units of the enzyme in 1 cc. (the reciprocal of the values given in Column 4 of Table I), as in the case of *p*-cresol, the total oxygen uptake at the end of 60 minutes was used as the measure of the activities of the preparations. In Fig. 2 are shown the

oxygen uptakes with time for three alumina-dialyzed preparations (Nos. E-I, E-II, and E-III) with catechol as the substrate. Upon comparing the total oxygen uptake at the end of 60 minutes (data taken from the curves in Fig. 2), it is found to be 430 c.mm. for Preparation E-I; 540 c.mm. for Preparation E-II; and 660 c.mm. for Preparation E-III. When these activities are put on a percentage basis, they become: Preparation E-I 65 per cent, Preparation E-II 82 per cent, and Preparation E-III 100 per cent. By arranging the activities of the same enzyme preparations towards *p*-cresol as the substrate (values taken from the last section in Table I) on a percentage basis, it is found that here too the three preparations show the same relative activities; Preparation

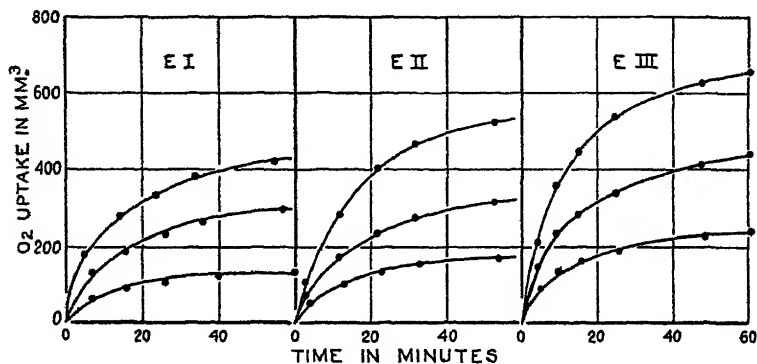


FIG 2. The oxygen uptakes plotted against time for three alumina-dialyzed preparations, with catechol as the substrate

E-I 67 per cent, Preparation E-II 82 per cent, and Preparation E-III 100 per cent. In other words, the three alumina-dialyzed preparations of tyrosinase from mushrooms show the same relative activities towards both the monohydroxyphenol, *p*-cresol, and the dihydroxyphenol, catechol.

From this similarity in activities of the tyrosinase towards the two substrates, the evidence is that we are dealing with an enzyme which activates oxygen in both cases. This point has significance with regard to Wieland's theory of dehydrogenation (7, 8). At least in the case of catechol it can be said that even though it appears that 2 hydrogen atoms are being removed in the formation of the *o*-quinone, it is the oxygen that is being activated.

## SUMMARY

1. A method for measuring quantitatively the activity of tyrosinase in terms of units is described.

2 Evidence is presented showing that the enzyme which catalyzes the oxidation of *p*-cresol is identical with the one which dehydrogenates catechol

3. Values are given for dry weight per unit of tyrosinase after each treatment in the processes of purification of the enzyme, as well as the loss in activity subsequent to each treatment in the purification of the enzyme.

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## A TUNGSTIC ACID PRECIPITATION METHOD FOR THE EXTRACTION OF ESTROGENIC SUBSTANCE FROM URINE

By S. CHARLES FREED, I. ARTHUR MIRSKY, AND SAMUEL SOSKIN

*(From the Department of Metabolism and Endocrinology, Michael Reese  
Hospital, Chicago)*

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The estimation of small amounts of estrogenic substance in the urine necessitates its extraction from relatively large volumes of fluid. This is generally accomplished by subjecting the urine to a continuous extraction with one of a number of fat solvents. This procedure requires the use of special apparatus and fairly large amounts of solvents; it is time-consuming, expensive, and hardly suitable for use in the routine laboratory.

In order to eliminate these difficulties, we have developed a much simpler procedure. The principle of our method is based on the previous observation that the closely related substance, cholesterol, can be removed quantitatively from the tungstic acid precipitate of normal or pathological urine (1).

### *Method*

Our procedure for the extraction of estrogenic substance from urine is as follows: To each liter of urine 50 cc. of 50 per cent sodium tungstate are added. This mixture is acidified with concentrated sulfuric acid<sup>1</sup> to a pH of 2 to 3, Congo red paper being used as indicator. To the acidified solution, 50 cc. of 33 N

<sup>1</sup> Sulfuric acid is particularly suitable for our method in view of the possibility that acid hydrolysis might result in greater estrogenic activity. As Cuyler (2) has recently shown, this acid exerts the least effect in this regard of all those which he tested, including the acids commonly used for the continuous extraction method. In our method the precipitate remains in contact with the acid at room temperature for an hour at most. We have boiled urine brought to a pH of 2 to 3 with sulfuric acid for several hours without changing the estrogenic activity obtained on assay.

sulfuric acid are slowly added and a heavy precipitate is formed.<sup>2</sup> This is allowed to settle for 30 minutes and then centrifuged until the supernatant fluid is clear. The latter is discarded. To the residue are added 100 cc. of an alcohol-ether mixture (3:1). The alcohol-ether mixture may be heated gently until it begins to boil, or it may be allowed to stand at room temperature for 1 hour without heating. Extraction is completed by either procedure. The mixture is then centrifuged and the alcohol-ether extract decanted into a beaker. The residue is washed with another small quantity of the alcohol-ether mixture, centrifuged, and the extract added to that in the beaker. The combined extracts are evaporated to dryness and the residue extracted twice with 25 cc. portions of anhydrous ether. 4 cc. of cottonseed oil are added to the ether extract and the mixture is heated gently until the ether is driven off, leaving the oil in which the estrogenic substance is dissolved. This oil is now ready for biological testing in rats or mice.

### *Results*

Adult spayed rats were used for our biological assays. The material to be tested was injected subcutaneously in two doses, administered 24 hours apart. 1 rat unit was considered to be contained in the least amount of total material so injected, which yielded a pure cornified vaginal smear within 48 to 72 hours after the first injection.

In order to estimate the efficiency of our extraction method, we first determined the estrogenic activity of samples of urine from pregnant women by injecting the untreated urine into rats. The same samples were also treated by the procedure described above and the estrogenic activity of the extract estimated in a similar manner. Table I details the results in eight such comparisons. In no instance was the activity of an extract less than that estimated by the direct method. In fact, the extracts frequently gave greater yields per cc. of urine than could be anticipated by assaying the untreated urine.

To test the accuracy with which various known amounts of

<sup>2</sup> When the 24 hour urine specimen is unusually dilute, so that it exceeds 1500 cc. in volume, it may be necessary to add more acid to obtain complete precipitation.

TABLE I

*Estrogenic Activity of Untreated Pregnancy Urine, Compared with Extracts of Same Urine*

Urine No	Rat units per cc of urine			
	Assay of native urine*		Assay of extract†	
1	>2 0	<3 0	>3 0	
2	>5 0	<10 0	>5 0	<10 0
3	>5 0	<10 0	>5 0	<10 0
4	>6 0	<12 0	>6 0	<12 0
5	>2 5	<5 0	>4 0	<12 0
6	>2 0	<6 0	>10 0	
7	>4 0	<8 0	>4 0	<8 0
8	>4 0	<8 0	>4 0	<8 0

\* Six to eight animals were used at each injection level

† Four animals were used at each injection level

TABLE II

*Recovery of Added Estrogenic Substance by Tungstic Acid Precipitation Method*

Urine No	Estrogenic substance (in urine) added to negative urine	Recovery of added estrogenic substance*
	rat units	rat units
1	1	>1
2	2	>2
3	2	>2
4	6	>6
5	8	>8
6	16	>16
7	16	16
8	16	16
9	16	16
10	16	16

\* In these assays the amount of extract calculated to contain 1 rat unit was injected. Where 1 or 2 units had been added, only one or two animals respectively could be used. Where greater amounts had been added, four animals were used in each case. Where > is used, strongly positive smears were obtained in practically every test. Where this sign is omitted, at least half the test animals gave positive results.

estrogenic substance could be extracted, a number of recovery experiments were done. To urine which demonstrated no estro-

genic activity, we added different amounts of pregnancy urine of known activity. The activity of extracts of this mixed urine was then estimated. Table II details some typical results and demonstrates that recovery of added estrogenic substance is uniformly complete within the biological error of animal assay.

In Table III we have tabulated some results obtained with our method on urines picked at random. The values that we have obtained are quite consistent with those obtained in similar cases by the continuous extraction method.

TABLE III

*Estrogenic Activity of 24 Hour Urines Extracted by Tungstic Acid Precipitation Method*

Urine No	Case	Estrogenic substance, rat units per 24 hrs		Urine No	Case	Estrogenic substance, rat units per 24 hrs	
1	Adult female	>2		9	Adult male	>2	<4
2	" "	>6		10	" "	>2	<4
3	" "	>6		11	Menopause	>1	
4	" "	>2	<4	12	"	>4	
5	" "	>8	<16	13	Acromegaly	>2	<6
6	" "	>16		14	10 yr. female		<1
7	" "	>16		15	10 " male	>2	
8	" "	>8	<16				

## DISCUSSION

It will be noted that our extracts frequently yielded somewhat higher assays than were obtained from corresponding amounts of untreated urine. Similar differences between the results of assay of untreated urine and of oil extracts of the same urine have been previously reported by Smith and Smith (3). They believed these differences to be due to the slower absorption of the oil solutions. This biological difference renders an exact recovery experiment difficult. Under these circumstances, and until more comparable techniques of assay have been devised, our results represent the closest possible approach to the demonstration of quantitative recovery of the estrogenic substance present in urine, by a method in which the final product of extraction is dissolved in oil.

## SUMMARY

A method is described for the quantitative extraction of small amounts of estrogenic substance from urine. The essential procedure is a preliminary precipitation with tungstic acid and extraction of the precipitate with alcohol and ether.

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## THE ISOLATION OF HOMOCYSTEINE AND ITS CONVERSION TO A THIOLACTONE

BY BYRON RIEGEL AND VINCENT DU VIGNEAUD

*(From the Department of Biochemistry, School of Medicine, George Washington University, Washington)*

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The interest that has been attached to the physiological behavior of homocystine, particularly its relationship to the intermediary metabolism of methionine and cystine, has directed attention to the reduced form of homocystine. We have therefore been interested for some time in the isolation of homocystine in order that certain of its chemical and physiological properties might be studied.

In considering the possible methods of isolating homocystine, the likelihood of the formation of a thiolactone had to be borne in mind. The presence of the —SH group in the  $\gamma$  position makes possible a thiolactone ring analogous to the lactone of  $\gamma$ -hydroxy acids. That such a ring closure can take place was demonstrated by Baernstein (1) who isolated the hydroiodide of the thiolactone of homocystine formed by the action of hydriodic acid on methionine, a reaction which we have been able to confirm, as we will discuss later. It was, therefore, not advisable to attempt to isolate homocystine by the methods often employed for cysteine in which the disulfide form is reduced with tin or zinc in hydrochloric acid and the reduced form isolated first as the hydrochloride. It was quite apparent that a more appropriate method of approach would be one involving an alkaline reduction, in which the homocystine would not be exposed to the action of acid for any appreciable length of time. Such a procedure as the sodium-liquid ammonia method used in the preparation of cysteine by du Vigneaud, Audrieth, and Loring (2) appeared to fulfil these requirements. From the fact that we had been able to prepare methionine by reduction of homocystine in liquid ammonia by metallic sodium



followed by methylation (3), we were aware that homocysteine could be converted to homocysteine by this method of reduction. We therefore took advantage of this reaction and we have succeeded in isolating the homocysteine in crystalline form as shown in Fig. 1.

The isolation of homocysteine made possible a study of the very interesting question of the ring closure of the compound. As mentioned above, we have been able to confirm Baernstein's isolation of the hydroiodide of the thiolactone by the action of hydriodic acid on methionine. In addition we have been able to prepare the same compound by the action of hydriodic acid on homocysteine, which is a further confirmation of the structure that was assigned by Baernstein to his compound.

For dealing with various phases of the chemistry of the lactone, the hydroiodide, however, is not particularly suitable. We have therefore tried to prepare some other salt of the lactone and have been able to isolate a beautifully crystalline hydrochloride, as shown in Fig. 2, by bringing about ring closure of the homocysteine with hydrochloric acid. The rate of ring closure was found to vary with the concentration of the HCl. For example, when homocysteine was refluxed with 0.1 N HCl, 50 per cent ring closure resulted in 3 hours, while heating it in 20 per cent HCl brought about practically complete conversion to the thiolactone within a few minutes. At room temperature with concentrated HCl 50 per cent lactonization resulted within 5 minutes and within 1 hour complete closure was obtained.

With  $\text{H}_2\text{SO}_4$  it was found that refluxing homocysteine with 18 N acid caused oxidation of the  $-\text{SH}$  to  $-\text{S}-\text{S}-$  to a much greater extent than it did ring closure. On the other hand, cold concentrated  $\text{H}_2\text{SO}_4$  caused about 8 times as much ring closure as oxidation. Refluxing the homocysteine with N or 6 N  $\text{H}_2\text{SO}_4$  brought about ring closure mainly, although some oxidation did occur. The experiments on ring closure were carried out in an atmosphere of nitrogen and the amount of  $-\text{SH}$  remaining at any given time was determined by iodine titration. The amount of disulfide formed was determined from the difference in the titration values for  $-\text{SH}$  before and after reduction.

As will be recalled, homocysteine was originally prepared from methionine by the action of 18 N  $\text{H}_2\text{SO}_4$  (4) and it was suggested

that demethylation had occurred with subsequent oxidation of the homocysteine so formed to the disulfide. On the other hand, as found by Baernstein, demethylation of methionine with hydriodic acid was found to lead to the formation of the lactone. It was therefore rather puzzling how one could obtain homocystine from methionine with sulfuric acid if homocysteine were formed in the reaction, as one might expect ring closure to take place in the acid medium. The oxidation, however, of homocysteine to homocystine with  $18\text{ N H}_2\text{SO}_4$ , as reported above, makes compre-



FIG 1

FIG 1. Homocysteine crystals.  $\times 50$ .



FIG 2

FIG 2 Crystals of the thiolactone hydrochloride of homocysteine  $\times 100$ . From absolute alcohol.

hensible the formation of homocystine from methionine with homocysteine as an intermediate

#### EXPERIMENTAL

*Preparation of Homocysteine*—A 500 cc three neck, round bottom flask fitted with a mercury seal stirrer, a calcium chloride tube containing soda-lime, and an inlet tube for the ammonia, was immersed in a large Dewar flask containing solid  $\text{CO}_2$  and trichloroethylene. 25 gm of S-benzylhomocysteine prepared according to the method previously described (5) were placed in the flask and ammonia distilled from sodium was run into the flask until about

400 cc of liquid ammonia were added. Since the S-benzylhomocysteine is more soluble near the boiling point of the ammonia, the temperature of the solution was kept very close to the boiling point. Small amounts of clean c.p. metallic sodium were added until a blue color resulted, which was permanent for 5 to 10 minutes. About 5.2 gm of sodium were necessary. Enough ammonium iodide was then added to dispel the blue color. The ammonia was then allowed to distil and most of the residual ammonia in the flask was removed by evacuation, care being taken to prevent any contact with oxygen. The flask was then flushed out several times with pure nitrogen. 50 cc of freshly boiled distilled water were then pipetted into the flask and the solution cooled in an ice bath. 45 per cent hydriodic acid was then added carefully until the solution was just acid to litmus. About 70 cc of the 45 per cent hydriodic acid were required. The solution was pinkish brown in color and held in suspension some dibenzyl which resulted from the debenzylation process. A small amount of norit was added and the solution filtered in such a manner as to be only in contact with pure nitrogen throughout the process. To the filtrate 500 cc of absolute alcohol were added and the flask allowed to stand in the ice box overnight. The homocysteine separated out in beautiful large flat crystals. The precipitate was filtered out of contact with air and washed with absolute alcohol and pure ether. It is necessary that both the alcohol and ether should be peroxide-, aldehyde-, and air-free. The homocysteine so prepared was dried over  $P_2O_5$  at a low pressure in nitrogen. 12 gm of the product were obtained, which was 80 per cent of the theoretical yield.

The above product, although beautifully crystalline and halogen-free, contained a small amount of disulfide, by titration of the sulfhydryl content by the Okuda method a sulfhydryl content of 92 per cent was obtained.

3 gm. of the product were dissolved in 30 cc of freshly boiled water, cooled, and kept under nitrogen. 100 cc. of absolute alcohol were then added and the homocysteine that precipitated was filtered immediately with the exclusion of oxygen and the filtrate made up to 500 cc with absolute alcohol. After standing overnight in the ice box the second fraction was filtered. It melted at 232-233°

(corrected) and after being dried at a reduced pressure in an atmosphere of nitrogen, the product gave the following analysis.

2.741 mg. substance: 0.243 cc.  $N_2$  at  $23^\circ$  and 771 mm.  
 4.581 " " : 7.889 mg.  $BaSO_4$  (micro-Parr bomb)  
 62.3 " " : 22.81 cc.  $KIO_3$  (1 cc. = 2.310 mg. cysteine, Okuda method)

$C_4H_7NO_2S$ . Calculated. N 10.37, S 23.72, —SH 24.4  
 Found. " 10.37, " 23.65, " 23.6

*Preparation of Homocysteine Thiolactone Hydrochloride*—5 gm. of homocystine were dissolved in 50 cc. of 6 N HCl. Granulated tin was then added and a few drops of 1 per cent platinum chloride. The mixture was heated on a water bath for 2 to 3 hours and then diluted to 250 cc. with hot boiled water.  $H_2S$  was passed into the solution and the stannous sulfide filtered. The filtrate was concentrated *in vacuo* in an atmosphere of nitrogen. Concentrated HCl was added and the solution again concentrated. This was repeated again and upon the concentration of the solution the hydrochloride of the thiolactone of homocysteine crystallized. The product was recrystallized from alcohol and ether. 4.6 gm. of the material were obtained, representing 80 per cent of the theoretical yield. The compound melted at  $200-201^\circ$  (corrected) and gave the following analysis.

2.870 mg. substance: 0.226 cc.  $N_2$  at  $26^\circ$  and 763 mm. (micro-Dumas)  
 0.0303 gm. " : 0.0461 gm.  $BaSO_4$   
 0.1044 " " : 0.0974 " AgCl

$C_4H_7NOS \cdot HCl$ . Calculated. N 9.12, S 20.88, Cl 23.08  
 Found. " 9.02, " 20.90, " 23.08

*Preparation of Homocysteine Thiolactone Hydrochloride from S-Benzylhomocysteine*—50 gm. of S-benzylhomocysteine were reduced by the method just described for its reduction in the preparation of homocysteine. After the evaporation of the ammonia the flask was evacuated, a current of nitrogen was passed through the flask, and 50 cc. of cool, freshly boiled water were added quickly. The solution was then subjected to reduced pressure to remove as much of the remaining ammonia as possible. 200 cc. of concentrated HCl were added and the flask heated on a boiling water bath for 1 hour. After this period of heating the solution was concentrated

*in vacuo*, 100 cc. more of concentrated HCl were added, and the solution again evaporated. 200 cc. of absolute alcohol were added to the residue and the solution concentrated to dryness to remove the remaining moisture. This was done once more and the dry salts were then repeatedly extracted with small portions of boiling absolute alcohol until the thiolactone was removed. The combined extracts were chilled in an ice bath and about 27 gm of the thiolactone hydrochloride melting at 197–200° were obtained, representing approximately 80 per cent of the theoretical yield. The product was further purified by recrystallization from ethyl alcohol.

#### SUMMARY

The isolation of homocysteine in crystalline form has been reported.

The conversion of methionine by hydriodic acid to the thiolactone of homocysteine has been confirmed.

A crystalline hydrochloride of the thiolactone of homocysteine has been prepared by the ring closure of homocysteine with hydrochloric acid

Homocysteine was found to be oxidized to homocystine by 18 N sulfuric acid, which makes more understandable the formation of homocystine from methionine by the action of 18 N sulfuric acid. With weaker concentrations of sulfuric acid ring closure was found to predominate

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# THE INFLUENCE OF THE INGESTION OF RAW PANCREAS UPON THE BLOOD LIPIDS OF COMPLETELY DEPAN- CREATIZED DOGS MAINTAINED WITH INSULIN\*

BY I. L. CHAIKOFF AND A. KAPLAN

(From the Division of Physiology, University of California Medical School,  
Berkeley)

(Received for publication, July 27, 1935)

In previous communications (1, 2) it was shown that in completely depancreatized dogs maintained for long periods of time with insulin and a diet containing meat, sucrose, bone ash, and vitamin supplements, there occurred a pronounced drop in all lipid constituents of the blood. The most marked change was found in the cholesterol fraction, the esterified portion of which was reduced to very low levels. In contrast to these changes in the blood, the lipid content of the liver was greatly increased, occupying up to 46 per cent of the wet weight of this organ. In an attempt to throw further light on the mechanism by which these lipid changes are produced, it seemed of interest to investigate the effects of the addition of raw pancreas to the diets of these animals. The results obtained for blood lipids are embodied in the present report.

## EXPERIMENTAL

Female dogs were used throughout in this investigation. The care of the depancreatized dogs has been described elsewhere (1). Two diets were employed: (1) in the first the animals received meat, sucrose, bone ash, and vitamin supplements in amounts

\* A preliminary report of a part of the results included in this paper appeared (*Proc. Soc. Exp. Biol. and Med.*, **32**, 934 (1935))

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TABLE I  
*Time of Onset of Cholesterol\* Changes in Completely Depancreatized Dogs Maintained with Insulin*

Dog	Weight	Preoperative					Days after pancreatectomy																							
		Total		Free		Ester		Total		Free		Ester		Total		Free		Ester		Total		Free		Ester						
		mg per 100 cc	mg per 100 cc	mg per 100 cc	mg per 100 cc	per cent of total	mg per 100 cc	mg per 100 cc	per cent of total	mg per 100 cc	mg per 100 cc	per cent of total	mg per 100 cc	mg per 100 cc	per cent of total	mg per 100 cc	mg per 100 cc	per cent of total	mg per 100 cc	mg per 100 cc	per cent of total	mg per 100 cc	mg per 100 cc	per cent of total	mg per 100 cc	mg per 100 cc	per cent of total			
	kg																													
D-H	9 6	154	120	34	22	21 days					67 days																			
						116	119	0	0	116	117	0	0	33 days					46 days											
						133	121	12	9	114	115	0	0	101	102	0	0	108	108	0	0									
D-L	9 0	140	120	29	19	34 days					41 days																			
						102	105	0	0	105	104	1	1	64 days					105 days											
						137	121	16	12	135	111	24	18	133	113	20	15	122	115	7	6									
D-15	8 0	150	118	32	21	8 days					34 days																			
						152	125	27	18	120	120	0	0	65 days																
						70 days																								
D-20	7 9	167	132	35	21																									
D-M	9 7	143	116	27	19																									
D-21	7 7	131	103	28	21																									

D-N	7 1	140	114	26	19	32 days			50 days			73 days					
						107	96	11	10	112	115	0	0	93	94	0	0
						82 days											
D-23	7 0	189	127	62	33	112	109	3	3								
						35 days				61 days				117 days			202 days
D-12	8 8	161	126	35	22	136	115	21	15	122	112	10	8	134	119	15	11
						122 days											
D-24	8 8	154	122	32	21	120	118	2	2								
						45 days				96 days							
D-25	9 7	167	117	50	30	140	126	14	10	145	131	14	10				

\* Whole blood was used for the analyses.



TABLE I  
*Time of Onset of Cholesterol\* Changes in Completely Depancreatized Dogs Maintained with Insulin*

Dog	Weight	Preoperative						Days after pancreatectomy																										
		Total			Free			Ester			Total			Free			Ester			Total			Free			Ester								
		mg per 100 cc	mg per 100 cc	per cent of total	mg per 100 cc	mg per 100 cc	per cent of total	mg per 100 cc	mg per 100 cc	per cent of total	mg per 100 cc	mg per 100 cc	per cent of total	mg per 100 cc	mg per 100 cc	per cent of total	mg per 100 cc	mg per 100 cc	per cent of total	mg per 100 cc	mg per 100 cc	per cent of total	mg per 100 cc	mg per 100 cc	per cent of total									
D-H	kg	154	120	34	22	21 days						67 days																						
						116	119	0	0		116	117	0	0																				
						12 days						20 days						33 days						46 days										
D-L	9 0	149	120	29	19	133	121	12	9		114	115	0	0						101	102	0	0				108	108	0	0				
						34 days						41 days																						
						102	105	0	0		105	104	1	1																				
D-15	8 0	150	118	32	21	34 days						50 days						64 days						105 days										
						137	121	16	12		135	111	24	18																				
						8 days						34 days						65 days																
D-20	7 9	167	132	35	21	152	125	27	18		120	120	0	0						104	105	0	0								122	115	7	6
						70 days						34 days																						
						132	133	0	0																									
D-M	9 7	143	116	27	19																													
D-21	7 7	131	103	28	21																													

D-N	7 1	140	114	26	19	32 days			50 days			73 days					
						107	96	11	10	112	115	0	0	93	94	0	0
						82 days											
D-23	7 0	189	127	62	33	112	109	3	3								
						36 days				61 days			117 days			202 days	
D-12	8 8	161	126	35	22	136	115	21	15	122	112	10	8	134	119	15	11
						122 days											9
D-24	8 8	154	122	32	21	120	118	2	2								
						45 days				96 days							
D-25	9 7	167	117	50	30	140	126	14	10	145	131	14	10				

\* Whole blood was used for the analyses.

previously specified (1, 2); (2) in studies in which the effects of raw beef pancreas were being observed the dogs received the glandular tissue twice daily in addition to the above constituents. The pancreas, stripped of adhering fat, was cut into small pieces (or occasionally ground) and mixed with the other dietary constituents at the time of feeding. The composition of a mixed sample of fifteen glands obtained by methods described elsewhere (2) is recorded in Table II.

*Whole blood* was used for lipid analyses and the oxidative methods employed have been previously noted (1). The animals were fed and injected with insulin twice daily at 8 00 a m and at 4 00 p m, while blood for analyses was taken just before the morning meal was served. Thus, at the time blood was sampled, all dogs were in the postabsorptive state, having ingested their last meal and received their last injection of insulin 16 hours prior to the removal of blood. Preoperative lipid determinations were made on postabsorptive blood obtained just before pancreatectomy.

*Time of Onset of Blood Lipid Changes in Completely Depancreatized Dogs Maintained with Insulin (Table I)*—Although the fall in blood lipids has already been noted, the interval after pancreatectomy, when the concentration of lipids in the blood began to change, remained to be determined. Since cholesterol, particularly the esterified portion, was most markedly affected by pancreatectomy, the cholesterol content of whole blood was taken as the index of the lipid changes. In the eleven dogs recorded in Table I, free and ester cholesterol were determined immediately before, and at repeated intervals between 8 and 202 days after excision of the pancreas. The preoperative values for cholesterol esters ranged between 26 and 62 mg. per 100 cc. of whole blood, or from 19 to 33 per cent of the total cholesterol. The lowest values obtained after pancreatectomy were between 0 and 10 mg. per cent. The results show that a drop below the preoperative level of cholesterol esters may occur as soon as 12 days and that this constituent may disappear from the blood as early as 20 days following pancreatectomy. The time at which these changes occurred, however, varied in the eleven dogs. Thus, while cholesterol esters were reduced to 0 values in six of the dogs at intervals between 20 and 70 days after removal of the gland, cholesterol esters were still present—although in greatly reduced amounts—

in the blood of Dogs D-12 and D-20 as late as 202 and 105 days respectively after pancreatectomy.

Although the free cholesterol content of the blood decreased after pancreatectomy, the changes produced in this constituent were

TABLE II

*Effect of Ingestion of Raw Pancreas upon Whole Blood Lipids of Completely Depancreatized Dogs Maintained with Insulin*

The dogs received 125 gm of raw pancreas\* twice daily in all diets fed after pancreatectomy.

Dog	Blood sample	Weight	Cholesterol				Total fatty acids	Phos-pho-lipid	Total lipid	Residual fatty acids†
			Total	Free	Ester					
		kg	mg per 100 cc	mg per 100 cc	mg per 100 cc	per cent of total	mg per 100 cc	mg per 100 cc	mg per 100 cc	mg per 100 cc
D-5	Preoperative	10 2	161	126	35	22	352	338	513	100
	68 days after pan- createctomy	10 7	287	141	146	51	633	422	920	244
D-11	Preoperative	9 4	145	112	33	23	348	248	493	158
	76 days after pan- createctomy	9 9	214	128	86	40	470	375	684	156
D-18	Preoperative	8 6	131	103	28	21	328	316	459	96
	76 days after pan- createctomy	9 2	229	124	105	46	544	438	773	173
D-O	Preoperative	13 5	143	113	30	21	324	293	467	106
	75 days after pan- createctomy	12 0	205	132	73	36	412	350	617	125
D-P	Preoperative	8 3	165	122	43	26	351	312	516	110
	75 days after pan- createctomy	7 5	289	137	152	53	474	442	763	67

\* Average analysis of raw pancreas: total cholesterol 0.22 per cent, phospholipid 1.87 per cent, total fatty acids 7.20 per cent, total lipid 8.62 per cent, water 73.8 per cent

† Fatty acids other than those in combination with phospholipids and cholesterol

not as great as those found in the esterified cholesterol. In seven of the animals pancreatectomy led to a decrease of 11 to 21 mg. of free cholesterol per 100 cc. of whole blood. In Dogs D-24 and D-H, practically no change in free cholesterol followed the excision of the gland, whereas in Dogs D-21 and D-25 a rise was observed.

*Blood Lipids of Completely Depancreatized Dogs Maintained with Insulin and Receiving Raw Pancreas in All Diets Fed after Pancreatectomy*—The five dogs in Table II received daily approximately 250 gm. of the glandular tissue in addition to the lean meat, sucrose, bone ash, and vitamin supplements already referred to. The ingestion of raw pancreas not only prevented the fall in blood lipids but led to a rise in all lipid constituents above preoperative values. The various lipid constituents did not share equally in this rise in blood lipids. In 68 to 76 days following pancreatectomy total fatty acids showed gains of 27 to 80 per cent above preoperative values. Although a gain of 62 to 126 mg. per 100 cc. above preoperative values occurred in total cholesterol under the influence of raw pancreas in the diet, only a small part, namely 15 to 21 mg., was due to free cholesterol. At an interval after pancreatectomy when, as shown by Table I, cholesterol esters were markedly reduced in the blood of completely depancreatized dogs ingesting no raw pancreas, the inclusion of the glandular tissue in the diets of the animals of Table II led to increases in cholesterol esters amounting to 143–317 per cent above preoperative levels. In Dogs D-5 and D-P the esterified cholesterol rose from preoperative values of 35 and 43 to 146 and 152 mg. per cent respectively. The least gain in this blood constituent occurred in Dog D-O, but even in this animal the concentration of cholesterol esters in the blood more than doubled 75 days after pancreatectomy. The phospholipid content of whole blood also rose above preoperative levels in the dogs that had received raw pancreas after pancreatectomy. Increases between 57 and 130 mg per 100 cc. of blood were observed in this constituent.

*Effect of Ingestion of Raw Pancreas upon Lowered Blood Lipids Produced by Pancreatectomy (Table III)*—Although raw pancreas in the diet prevented the fall in blood lipids that was found to ensue after pancreatectomy (Tables I and II), the question arose whether this glandular tissue was still effective after low lipid levels had been established. That even under such abnormal conditions raw pancreas can raise the lipid concentration is shown by Table III. The postoperative period of observation in these animals may be divided into two parts: the first, a period in which no pancreas was included in the diet, and the second, in which these dogs received the glandular tissue twice daily. At the end of the

TABLE III

*Effect of Ingestion of Raw Pancreas upon Lowered Blood\* Lipids Produced by Pancreatectomy*

Dog	Weight	Blood sample	Duration of raw pan- creas in diet when blood sample was taken	Cholesterol				Total fatty acids	Phospho- lipid	Total lipid	Residual fatty acids
				Total	Free	Ester					
	kg		days	mg per 100 cc	mg per 100 cc	mg per 100 cc	per cent of total	mg per 100 cc	mg per 100 cc	mg per 100 cc	mg per 100 cc
D-20	7 9	Preoperative		167	132	35	21	395	340	562	141
	7 8	105 days after pancreatec- tomy	0	122	115	7	6	265	260	387	86
	8 7	139 days after pancreatec- tomy	34†	222	133	89	40	492	363	714	184
D-12	8 8	Preoperative		161	126	35	22				
	7 8	202 days after pancreatec- tomy	0	128	116	12	9	363	295	491	156
	8 5	243 days after pancreatec- tomy	41‡	265	128	137	52	593	486	858	168
D-E	6 6	838 days after pancreatec- tomy	0	123	119	4	3	294	253	417	121
	8 7	940 days after pancreatec- tomy	102‡	241	137	104	43	538	448	779	162
D-24	8 8	Preoperative		154	122	32	21	356	326	510	115
	8 5	122 days after pancreatec- tomy	0	120	118	2	2	262	235	382	104
	9 0	162 days after pancreatec- tomy	40‡	243	146	97	40	547	424	790	192
D-25	9 7	Preoperative		167	117	50	30	342	329	509	85
	9 9	96 days after pancreatec- tomy	0	145	131	14	10	326	283	471	126
	10 8	130 days after pancreatec- tomy	34‡	258	137	121	47	571	476	829	164

\* Whole blood was used for the analyses.

† 70 gm. of raw pancreas twice daily.

‡ 125 gm. of raw pancreas twice daily.

first period the cholesterol esters of the blood had fallen to 2 to 14 mg. per cent in Dogs D-20, D-12, D-25, and D-24, whereas after the addition of raw pancreas to their diets cholesterol esters rose from these low values to values between 89 and 137 mg per cent. At 385 and 448 days after pancreatectomy 1 and 0 mg per cent of cholesterol esters were found in the blood of Dog D-E (1). It is again interesting to note that at 838 days after excision of the gland the cholesterol ester content of the blood was still low; namely, 4 mg per cent. Despite the prolonged reduction of this constituent in the blood, the addition of raw pancreas to the diet for 102 days produced an increase in cholesterol esters from 4 to 104 mg. per 100 cc. of blood or from 3 to 43 per cent of the total cholesterol.

Similar changes were produced in total fatty acids and phospholipids. Both constituents had fallen below preoperative levels at the end of the first period of observation during which none of the glandular tissue had been included in the diet. The ingestion of raw pancreas during the second period led to increases in phospholipids and total fatty acids, the final values attained being far in excess of the preoperative

*Response of Blood Lipids of Depancreatized Dogs to Alternate Addition and Removal of Raw Pancreas from Diet*—In three dogs a study was made of the effects produced in blood lipids by alternately adding and withholding raw pancreas from the diet. A typical result is shown in Chart 1, which clearly demonstrates the readiness with which total fatty acids, phospholipids, and cholesterol esters respond to the presence and absence of the glandular tissue in the diet. Although high lipid levels are easily established by the ingestion of raw pancreas, they cannot be maintained unless the glandular tissue is continually included in the diet; completely withholding raw pancreas results in a rapid and pronounced drop from a previously established high level to one below normal.

During the rise in blood lipids produced by the ingestion of raw pancreas, total cholesterol values between 200 and 300 mg. per cent were observed. Such high concentrations were attained irrespective of whether the lipid rise began from preoperative levels (as in the case of feeding raw pancreas to dogs immediately after pancreatectomy) or whether the rise was effected after low lipid levels had first been established by withholding the glandular

tissue after pancreatectomy. The relation of the time factor in these changes is also worthy of note. After 59 days of the gland feeding, total cholesterol in Dog D-P (Chart 1) rose to 292 mg. per

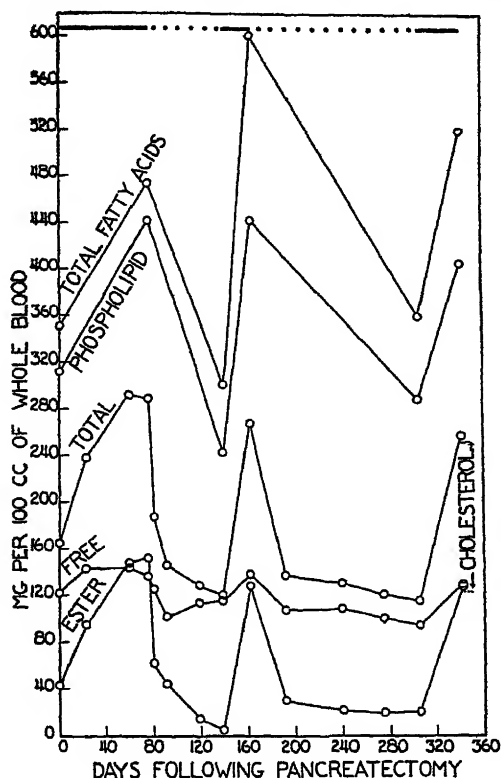


CHART 1. The response of blood lipids of Dog D-P (completely depancreatized and maintained with insulin) to the addition and removal of raw pancreas from the diet. The heavy lines show the periods in which 250 gm. of raw pancreas were included in the diet daily. The dotted lines represent the intervals during which the diet mixtures contained none of the glandular tissue. The preoperative values for blood lipids are recorded on the zero day.

cent. The continuation of pancreas feeding for 16 more days failed to increase the cholesterol content of the blood above this level. In Dog D-E (Table III) total cholesterol rose to 241 mg.



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per cent after 102 days of the gland feeding, whereas in three other dogs recorded in the same table values of 222 to 265 mg were observed after 34 to 41 days of pancreas ingestion. It would seem that under the conditions of the present study the maximum concentration of total cholesterol that can be produced by pancreas feeding is below 300 mg. per cent.

### DISCUSSION AND SUMMARY

The results of the present investigation show that the level of the blood lipids of the completely depancreatized dog maintained with insulin can be strikingly influenced by the presence and absence of raw pancreas in the diet. When such animals are fed a diet containing none of the glandular tissue, a drop in the lipid concentration of the blood sets in soon after pancreatectomy. The time of onset of these changes in blood lipids was found to vary among the animals studied. A drop below the normal or pre-operative level occurred as early as 12 days after pancreatectomy. In eight dogs cholesterol esters were reduced to between 0 and 3 mg per cent at intervals of 20 to 122 days following pancreatectomy, while in three others 7 to 14 mg. per cent were still found in whole blood as late as 96 to 202 days after pancreatectomy.

The addition of raw pancreas to the diets of depancreatized dogs being kept with insulin produced an entirely different blood lipid picture. The various effects observed may be summarized as follows:

- 1 When raw pancreas was included in all diets fed after pancreatectomy, a rise instead of a fall in whole blood lipids occurred

- 2 The addition of raw pancreas to the diets of depancreatized dogs in which a low lipid level had first been established resulted in a rapid and pronounced rise in all lipid constituents. The rise was particularly striking in the case of cholesterol esters. Thus, in one of the dogs studied (Chart 1) cholesterol esters rose from 5 to 129 mg. per cent in a period of 23 days at the same time that the concentration of total fatty acids and phospholipids almost doubled

3. In dogs in which raw pancreas was included in all diet mixtures fed after pancreatectomy, the concentration of all blood lipid constituents was always in excess of the preoperative level. As a

result of the ingestion of the glandular tissue, total fatty acids rose from preoperative or normal values of 324 to 352 mg. per cent to 412 to 633 mg. per cent and cholesterol esters from 28 to 43 mg. per cent to 73 to 152 mg. per cent. Even in those animals in which raw pancreas was added to the diets only after the blood lipids had already fallen considerably below preoperative values, the lipid levels finally attained were much higher than the preoperative.

4. The removal of raw pancreas from the diet at a time when high blood lipid levels had been produced by its ingestion resulted in a rapid and pronounced drop in blood lipids. All lipid constituents participated in the fall as they had in the rise. High blood lipid levels in the completely depancreatized dog kept with insulin are found only so long as raw pancreas is being ingested.

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## ANALYSIS OF TYPE I PNEUMOCOCCUS SPECIFIC PRECIPITATE

BY HERBERT O. CALVERY

*(From the Department of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor)*

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Since the antibodies of an immune animal are associated with the globulin fraction of the serum proteins, it is very difficult by the usual methods of preparation to obtain antibodies which are not contaminated in various degrees with other serum proteins. For this reason very few chemical investigations have been made of what were considered relatively pure antibodies.

In a previous publication an analysis of Type II pneumococcus specific precipitate was presented (1). Hewitt (2) has recently made an investigation of diphtheria toxin-antitoxin floccules in which he reported the amide N, monoamino N, diamino N, cystine, tyrosine, and tryptophane. These values were compared with the values for the same constituents in the crystalline albumin fraction and the globulin fraction. By the chemical methods which he used no marked differentiation of these materials from each other was possible.

As is well known when the specific haptens of Type I and Type II pneumococcus, which are carbohydrate in nature, are added to antisera, a heavy precipitate is formed which is quite insoluble and can be carefully washed and dried. It seemed quite possible that such a precipitate should contain the antibody protein in the purest form available, since the hapten is non-protein in nature and the non-specific serum proteins could be washed out by means of dilute salt solutions and water. Through the kindness of Dr. M. Heidelberger and Dr. F. E. Kendall of Columbia University, the author obtained a quantity of such purified precipitate from Type I specific hapten and antisera which has been analyzed by the methods in general use in this laboratory (3) and the results are

reported in Table I. The specific precipitate contained about 5 per cent of the specific carbohydrate and the results have not been corrected for this value. They are, therefore, directly comparable with the values previously reported (1) for Type II specific precipitate. The results of two analyses of 3 gm of protein are reported in Table I, and for comparison an average of the results previously reported for Type II specific precipitate are also included

TABLE I  
*Analytical Values for Type I and Type II Pneumococcus Specific Precipitates*

All values are corrected for ash and moisture. The nitrogen fractions are expressed as percentages of the total nitrogen

	Type I		Type II*
	Sample I	Sample II	
Ash . . . .	0 26	0 28	0 17
Moisture . . . .	4 9	4 8	6 1
Total N . . . .	15 8	15 9	16 0
Amide " . . . .	4 3	4 7	3 7
Humin " . . . .	0 6	0 6	0 6
Amino " (after hydrolysis)	74 1	76 3	75 0
Phosphorus . . . .	None	None	None
Sulfur . . . . .	1 2	1 3	1 3
Tyrosine . . . . .	5 6	5 4	5 5
Tryptophane . . . .	2 1	2 0	2 2
Cystine . . . . .	2 5	2 6	3 1
Arginine . . . . .	4 9	5 0	5 5
Histidine . . . . .	0 9	1 0	1 1
Lysine . . . . .	5 4	5 3	4 8
Aspartic acid . . . .	4 7	4 5	4 4
Glutamic " . . . .	6 3	6 8	6 3

\* The values are averages of the analyses previously reported (1)

The results agree very closely with those obtained for Type II specific precipitate (1). The only difference is in the cystine value which for Type II was 3.1 per cent and for Type I is 2.5 per cent. The difference between these is beyond experimental error, while all other values for both substances resemble duplicate analyses of the same substance.

In a general way the values agree with those reported in the

literature for serum globulins but specific comparisons are almost impossible since no single globulin fraction has been analyzed for all the constituents reported here. The cystine and tryptophane values are somewhat higher and the tyrosine values slightly lower than those reported by Hewitt (2) for diphtheria toxin-antitoxin floccules, for which amino acids the mean values were 2.05, 1.80, and 5.85 per cent respectively. From the comparative values obtained in these investigations it seems probable that further careful and complete analyses of highly purified antibodies will afford valuable information concerning their chemical composition, their probable method of formation, and the nature of the reactions between antigens and antibodies.

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## CRYSTALLINE EGG ALBUMIN\*

### II. THE FRACTIONATION OF PEPTIC HYDROLYSIS PRODUCTS

By HERBERT O. CALVERY†

*(From the Department of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor)*

(Received for publication, August 19, 1935)

In a previous investigation of the series (1) the importance of a study of the action of enzymes on crystalline egg albumin was emphasized and references to the early literature were cited. The former results showed quite conclusively that when crystalline egg albumin was treated with pepsin approximately one-third of the peptide linkages in the molecule were hydrolyzed in from 8 to 40 days, depending on the amount of pepsin used. One of two conclusions could be drawn from this. Either tripeptides were the end-products of peptic digestion of egg albumin or if longer chain peptides were formed it was inevitable that simpler products such as dipeptides and possibly free amino acids were liberated in order to account for the number of peptide linkages broken. The present paper is a report of a fractionation of the peptic hydrolysis products of egg albumin and of the information obtained by chemical and enzymatic studies of these fractions.

#### EXPERIMENTAL

50 gm. of crystalline egg albumin and 10 gm. of pepsin (Difco, 1:20,000) were placed in a liter flask and made to volume with 0.3 per cent hydrochloric acid. A control with 10 gm. of pepsin was run simultaneously and all results reported have been corrected for the pepsin present in the digest. After 20 days the amino nitrogen liberated was equivalent to 25.15 per cent of the total

\* Paper I of this series is reference (1) of the bibliography.

† The author wishes to express his appreciation for a grant from the Faculty Research Fund of the University of Michigan, which made this investigation possible.



nitrogen and 3 days later the same results were obtained, indicating that equilibrium had been attained. This was equivalent to approximately one-third of the peptide nitrogen and was in accord with previous results, hence more pepsin was not added.

The digestion was discontinued at this point by heating at 85° for 10 minutes and filtering from the small coagulum (300 mg.). The clear filtrate was concentrated *in vacuo* at 40° to 500 cc., cooled to 0°, and poured into 9 volumes of absolute alcohol. After standing a few days at 0°, 6 gm. of material were filtered off. The filtrate (Fraction A) was concentrated under reduced pressure at low temperature to remove the alcohol and the volume made up to 500 cc. The precipitate (Fraction B) was dissolved in water and the volume made up to 250 cc. The amino nitrogen of Fraction A was 30.05 per cent of the total nitrogen, while after acid hydrolysis the amino nitrogen was 77.9 per cent of the total nitrogen. In Fraction B the amino nitrogen was only 15.36 per cent of the total nitrogen and after acid hydrolysis the amino nitrogen was 79.0 per cent of the total nitrogen. Fraction B obviously contains longer chain peptides than tripeptides and likewise Fraction A must contain some shorter chains and possibly some free amino acids.

Further fractionation of Fraction B was obtained by treatment of the solution with an excess of 10 per cent flavianic acid. A heavy oil separated which dissolved when the solution was heated but appeared again when the solution was cooled. After standing at 0° for several days, the supernatant liquid (Fraction B<sub>F</sub>) was decanted from the oil (Fraction B<sub>P</sub>). Fraction B<sub>P</sub> was dissolved in 0.2 N sulfuric acid and sulfuric acid added to Fraction B<sub>F</sub> to 0.2 N concentration. The flavianic acid was quantitatively removed from both fractions by extraction with purified butyl alcohol. The amino nitrogen of Fractions B<sub>P</sub> and B<sub>F</sub> was 16.5 per cent and 14.4 per cent, while the arginine nitrogen determined by isolation of the flavianate after acid hydrolysis was 11.55 per cent and 9.35 per cent respectively of the total nitrogen.

Fraction A was neutralized to pH 7 and extracted in a continuous extractor with butyl alcohol under reduced pressure at 35° for 48 hours. Three fractions were obtained: Fraction A<sub>1</sub>, the material not extracted with butyl alcohol; Fraction A<sub>2</sub>, the material extracted with butyl alcohol but insoluble in absolute ethyl alcohol;

and Fraction A<sub>3</sub>, the material extracted with butyl alcohol and soluble in absolute ethyl alcohol. Fraction A<sub>1</sub> was equivalent to 70 per cent of the original Fraction A, contained 26.4 per cent of the total nitrogen as amino nitrogen, 2.0 per cent tyrosine, 0.74 per cent tryptophane, and, after acid hydrolysis, 78.1 per cent of the total nitrogen was amino nitrogen. Fraction A<sub>2</sub> (3.3 gm. of a non-hygroscopic white powder) contained 61.1 per cent of the total nitrogen as amino nitrogen, 4.6 per cent tyrosine, 1.3 per cent tryptophane, and, after acid hydrolysis, 71 per cent of the total nitrogen was amino nitrogen. Fraction A<sub>3</sub> was equivalent to 20 per cent of the original Fraction A, contained 34.5 per cent of the total nitrogen as amino nitrogen, 6.9 per cent tyrosine, 2.5 per cent tryptophane, and, after acid hydrolysis, 85 per cent of the

TABLE I  
*Increase in Amino N Expressed As Percentage of Total N Caused by Treatment of Materials with Respective Enzymes and Acids*

	Fraction A <sub>1</sub>	Fraction A <sub>2</sub>	Fraction A <sub>3</sub>	Fraction B <sub>1</sub>	Fraction B <sub>2</sub>
Erepsin	49.5	11.1	46.8	45.2	63.8
Aminopolypeptidase	36.8	7.0	35.2	38.2	54.6
Dipeptidase	12.0	4.6	10.4	5.3	8.2
Protaminase	None	None	None	8.8	None
Acid hydrolysis	52.3	10.0	50.3	48.0	65.2
Total amino N (after acid hydrolysis)	78.1	71.0	85.0	64.5	79.5

total nitrogen was amino nitrogen. The results obtained by the action of enzymes on the various fractions are recorded in Table I. The dipeptidase was prepared according to the method of Macrae (2) and the other enzymes by the methods previously described (1).

#### SUMMARY

The previous investigation which showed that approximately one-third of the peptide linkages of crystalline egg albumin were hydrolyzed with pepsin has been confirmed. The digestion products have been separated into five fractions and some chemical and enzymatic studies of these fractions show that they are quite different from each other. The fact that Fraction A<sub>2</sub> contains only 10 per cent peptide nitrogen leads to the inevitable conclusion

that free amino acids are present. Efforts are being made to identify some of the constituents of this fraction.

The results of the action of the specific dipeptidase recorded in Table I lead also to the conclusion that dipeptides are formed during peptic hydrolysis of egg albumin.

Results of further investigations by other methods of fractionation of which a preliminary report has already been made (3) will be reported in detail in the near future.

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## ACTIVATION OF ENZYMES

### III. THE RÔLE OF METAL IONS IN THE ACTIVATION OF ARGINASE. THE HYDROLYSIS OF ARGININE INDUCED BY CERTAIN METAL IONS WITH UREASE

BY LESLIE HELLERMAN AND MARIE E. PERKINS

(From the Department of Physiological Chemistry, the Johns Hopkins University, School of Medicine, Baltimore)

(Received for publication, August 22, 1935)

Earlier papers from this laboratory have dealt with the activation of urease (1) and of papain (2), with emphasis, particularly, upon reversible inactivations by oxidizing agents and by certain organometallic compounds. This communication describes an investigation of the markedly differing activation chemistry of the enzyme, arginase. There is stressed the conspicuous rôle apparently played by metal ions in the arginase-arginine reaction, a study of which has disclosed the conditions under which hydrolysis of arginine may be extensive *when induced only by certain metal ions with urease*.

Although enzyme literature is replete with descriptions of the activating and inactivating effects of various reagents (*cf.* (3)), there have appeared only recently reports of investigations in which has been attained a measure of success in the correlation of some of these effects in terms of a rational chemistry. Progress has been impeded in part by the difficulty of obtaining most enzymes in a state of even approximate purity or in the reproduction by different workers of crude enzyme preparations possessing sufficiently constant behavior.

For our initial studies in this field we selected crystalline urease and papain, the properties of which promised relative freedom from these difficulties. Controlled inactivations of these enzymes by various oxidizing agents were found to be extensively reversible, the reversals being effected by a number of reducing substances. The inactivating effects of cuprous oxide and certain organic

mercury derivatives, which are known to be mercaptide-forming, were likewise shown to be reversed by suitable reagents. The results as a whole seemed to support the tentative hypothesis that for urease or papain such effects may largely be attributed to reversible chemical actions upon thiol groups which for the present may be assumed to be integral substituents of the enzyme molecules themselves<sup>1,2</sup> (1, 2).

While this simple and attractive picture may well reflect a phenomenon of considerable biochemical importance, it can by no means be regarded as universally applicable. This is sufficiently illustrated by a consideration of the diverse, and often contradictory, findings of many recent investigators of arginase activation. The earlier opinions of several investigators (8, 9) concerning the rôle of organic thiol compounds, *e g.* glutathione, as specific activators of arginase were abandoned when it was found (10) that the effects of such compounds are variable and that ferrous ion, alone or used together with sulfhydryl (11, 12) or with certain other organic compounds (13), is a much more characteristic activator. Little progress has as yet been made in defining arginase activation (14, 15) in terms of a reversible oxidation-reduction process, and there exists among various workers upon this aspect no agreement as to the significance of the results obtained.

Using a liver-enzyme preparation of satisfactory stability and potency, of a high degree of specificity<sup>3</sup> as arginase, and free of thiol compounds that respond to nitroprusside, we have ascertained that the arginase action neither is significantly altered by

<sup>1</sup> The work of Shwachman, Hellerman, and Cohen (4) disclosed a striking and perhaps significant analogy to these observations upon urease and papain in the effects of oxidation-reduction and mercaptide-forming agents upon the lytic action of hemolysin of pneumococci, Type II

<sup>2</sup> The concept of enzyme activity control through reversible oxidative attack upon a grouping of the enzyme molecule was independently suggested by Bersin and Logemann (5) in a paper upon papain which appeared shortly after our communication upon urease and before our own experimental work with papain activation was completed. Maschmann and Helmert (6) suggested that the activation of cathepsins and of papain by HCN is related to the reduction to thiol of dithio groupings in the protein-enzyme molecules, and Bersin subsequently (7) interpreted his findings in terms of thiol chemistry

<sup>3</sup> Unpublished work of Chester Stock and the authors

mercaptide-forming organomercurials (*e.g.*  $\text{C}_6\text{H}_5\text{HgOH}$ ) nor is enhanced by sulfhydryl compounds like cysteine. Certain other reducing agents, *e.g.* bisulfites, cyanides, and hydrogen sulfide, which are also effective papain activators, likewise fail to activate this arginase. On the other hand, we have found that arginase activity is enhanced under certain conditions by any one of the ions,  $\text{Co}^{++}$ ,  $\text{Ni}^{++}$ , or  $\text{Mn}^{++}$ , as well as  $\text{Fe}^{++}$ . Indeed, the activity is apparently restored and brought to its maximum by the simple addition of cobaltous ion after treatment of the enzyme with such inhibitors as quinone, ferric ion, or iodine. Furthermore, arginase is suppressed by cyanide or by hydrogen sulfide, although partial restoration from the action of the latter is readily effected by suitable removal of the excess reagent; and the activity is more completely reestablished by the subsequent addition of an appropriate "bivalent" metal ion. These observations reveal a striking contrast between the mode of activation of arginase, on the one hand, and of urease or papain, on the other. They bring into sharp focus the probable significance of metal ion chemistry for the arginase-arginine reaction.

This is of additional interest in view of the remarkable fact, disclosed by this work for the first time, so far as we are aware, that arginine (without arginase) is readily hydrolyzed, even at pH 7.5 at  $30^\circ$ , in the presence of certain metal ions ( $\text{Co}^{++}$ ,  $\text{Ni}^{++}$ ,  $\text{Mn}^{++}$ ) *when urease (crude jack bean or the crystalline protein enzyme) is also present in the reaction mixture*. Quite apart from its inherent theoretical interest, the latter discovery requires consideration in connection with certain analytical operations in addition to those actually concerned with the study of arginase catalysis.

#### Methods

*Arginase Preparation*<sup>4</sup>—The freshly excised liver of a calf is cut into small pieces which are placed in crushed ice, in a glass container. The chilled tissue (about 2 pounds) is ground and mixed well during 1 hour with 300 ml. of glycerol (U.S.P.). The mixture is pressed through several layers of muslin (in a meat press), yielding an extract of perhaps 50 ml. For present purposes this is discarded, and to the *residue* from this extract are added 500 ml. of water. This mixture is stirred  $\frac{1}{2}$  to 1 hour, after which it is

<sup>4</sup> Cf. (16).

pressed through muslin, yielding an extract of about 220 ml. The residue may be profitably reextracted and worked up further, if desired. To the *fluid extract* is added toluene, 25 ml., and, after being well shaken, the mixture is allowed to stand in a refrigerator for 12 hours. It is then centrifuged 1 to 1½ hours (at 2400 R.P.M.). The top toluene-fat layer and a small amount of solid on the bottom are discarded. To the somewhat cloudy middle layer is added acetone (U.S.P.) until the precipitation of solid material is complete. After the precipitate has settled, most of the yellow supernatant liquid is removed by decantation; the solid is collected on Buchner funnels and washed well with acetone (C.P.). It is air-dried on sheets of filter paper for removal of residual acetone, after which it is dried in a vacuum desiccator over  $P_2O_5$ . This crude enzyme powder (about 13 gm.) may be kept in a glass-stoppered bottle at room temperature. Aqueous extracts of such material provided the arginase used in this work. As implied in the foregoing, continued extraction of the liver tissue with appropriate subsequent manipulations yields more crude arginase. The details were worked out with the aid of determinations of enzyme activity upon all fractions and this guided the selection of the fraction actually used.

*Arginase Solution*—A solution of enzyme is freshly prepared by addition of 5 mg. of the solid preparation to each ml. of redistilled water taken; the mixture is stirred, heated at 37° for 1 hour, and filtered. The residue is discarded. The clear filtrate contains the enzyme activity and will be called hereafter *arginase solution*.

Such extracts have not yet been fully examined chemically or spectrographically. Coagulable protein is present; compounds that respond to the nitroprusside test are absent; extracts, heated with alkaline plumbite, yield PbS. It may be noted that this enzyme preparation has been found to be rather highly specific as arginase. Work by Chester Stock and the authors (to be described in a later communication) has demonstrated that this arginase fails to catalyze the hydrolysis of  $\alpha$ -hydroxy- $\delta$ -guanidovaleric acid (argininic acid) or  $\delta$ -guanidovaleric acid under conditions which permit the extensive hydrolysis of arginine.

*Determination of Arginase Activity*—The development of a quantitative method suited to this problem was aided by reference to the work of Jansen (17), Edlbacher (18), and Hunter (19) and

their collaborators. The urea formed by hydrolysis of arginine was determined essentially by Marshall's method (20) as adapted to precision work by Van Slyke and Cullen (21). There follows a description of a *typical experiment*.

Water redistilled in glass apparatus was used throughout. Arginase solution was prepared from some arginase powder which had stood for several months. To 2 ml. of this, contained in a 25 ml. volumetric flask, were added 8 ml. of water and 5 ml. of 1 M phosphate buffer, pH 7.5. In a parallel determination there then were added 3 mg. of cobaltous chloride ( $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ). Each mixture stood 15 minutes (at  $25^\circ$ ), after which it was brought to  $37^\circ$  and treated with a few drops of caprylic alcohol and a solution of 45 mg. of arginine carbonate (or an equimolar amount of arginine hydrochloride) in 1 ml. of water. Hydrolysis was permitted to proceed at  $37^\circ$  for exactly 2 hours; the flask was plunged into boiling water and held there for 10 minutes, after which it was cooled to room temperature and the following additions made, in the order named: phosphate buffer (1 M, pH 7.5) 5 ml., sodium bisulfite 5 mg., sodium cyanide 16 mg., and 0.1 gm. of jack bean urease powder (22). The volume was brought to 25 ml., a few drops of caprylic alcohol added, and the mixture kept at  $30^\circ$  overnight, after which it was filtered. Of the aqueous filtrate, 10 ml. were added to 10 ml. of saturated  $\text{K}_2\text{CO}_3$  solution and a few drops of caprylic alcohol, and the ammonia carried into 0.02 N HCl by aeration for 2 hours. The excess HCl was titrated with 0.02 N NaOH, chlor-phenol red being used as indicator.

The results were as follows: *With  $\text{Co}^{++}$* , ammonia in 10 ml. was found equivalent to 6.70 ml. of HCl (0.02 N) after correction by subtraction of a blank (0.23 ml.) obtained in a control in which arginine alone was omitted; without  $\text{Co}^{++}$ , 3.65 ml. (corrected). In a control in which arginase was omitted ( $\text{Co}^{++}$  and arginine present) and urea, 10.0 mg., added immediately before the 2 hour incubation at  $37^\circ$ , the acid found equivalent to  $\text{NH}_3$  (from 10 ml. of filtrate) was 6.61 ml. (corrected), the *calculated* amount (*i.e.* equivalent to 4 mg. of urea) being 6.67 ml.; the correction was applied by subtraction of 0.47 ml., obtained in a similar control with urea absent.

*Discussion of Method*—The development of details of the method need not be discussed at length. Some of the factors considered



will be treated briefly. Arginase solution from recently prepared arginase powder was usually found to be more potent (when non-activated by  $\text{Co}^{++}$ , etc.) than the extracts of older preparations; however, the maximal activities (activated enzyme) remained fairly constant for months. Some variations are, therefore, to be anticipated in the activity of different crude preparations and in preparations of varying ages

In the typical determination (cited above), phosphate in the reaction mixture was about  $\text{m}/3$ . Increase of the phosphate concentration to  $\frac{2}{3} \text{ M}$ , other conditions being unchanged, resulted in a decline of about 15 per cent in apparent activity; enhancement of activity accompanied a decrease to 0.1  $\text{M}$  in the phosphate concentration. A systematic determination of the effect of ionic strength was not of *immediate* importance. In a series of experiments in which veronal (23) buffer (pH 7.5), present in a concentration of 0.07  $\text{M}$ , was substituted for phosphate buffer of the same pH and concentration, the enzyme activity was not significantly changed. However, specific and general salt effects may become of great moment, as is demonstrated by the observations of Hunter *et al.* (19) of the marked depression of the activity by borate in comparison with the effects of certain other buffers (pH, unchanged); similar results were obtained with this arginase. The relation of pH to arginase activity has already been evaluated (18, 19). For work at pH 9.5, we used Sorensen's glycine buffer, taking due account of the potential reactivity of glycine toward various reagents (*e g.* oxidizing agents). The arginine salts used were preparations from gelatin, made and purified in this laboratory, or high grade commercial products.

Sodium bisulfite (used especially when quinone had been previously added, and not necessarily otherwise) and sodium cyanide were added in order to protect urease against the action of various reagents; *e g.*, excess quinone, metal ions, etc (*cf.* (1, 2)). It was amply demonstrated by means of suitable controls that the accuracy of the urea estimations was not impaired. An additional, indispensable function of the cyanide will be discussed later.

Fosse's xanthidrol method (24) for the estimation of urea as adapted to arginase determinations by Karrer and Zehender (25), was found of some utility for qualitative purposes but of limited value for the quantitative needs of this work. Although no

precipitation of dioxanthidryl urea was obtained from mixtures in which hydrolysis of arginine had not taken place, the weight of this derivative obtained from hydrolyzed material was occasionally far in excess of that anticipated on the basis of parallel urease determinations. Such dioxanthidryl urea was found by analysis (N) to be impure and to contain ash.

#### DISCUSSION OF RESULTS

A detailed account of the numerous individual experiments underlying this work will not be attempted. A few representative data are tabulated to illustrate the magnitude of the effects observed under specified conditions. The experimentation has necessarily been guarded at every stage by adequate control determinations.

It should be emphasized at once that comparisons of the results of different investigators in this field must take account of the varying experimental conditions employed. Arginase preparations are at best crude mixtures, varying greatly in their content of extraneous material. This material contributes to the activation picture and modifies the effects of added reagents. The hydrogen ion concentration of the reaction mixture necessarily influences the action of many reagents. Specific effects of a buffer salt may not be predictable. For example, ferric ion or selenious oxide abolishes the activity of our preparation when added to the enzyme before, but not after, the addition of phosphate buffer (pH 7.5); cobaltous ion activates regardless of the order of addition. Much of the arginase work already reported rests upon determinations of activity in glycine buffer at pH 9 to 9.5, presumably because this is near the pH range of optimal activity for arginase. The preparation used by us was found sufficiently active in phosphate of pH 7.5, a less "favorable" region, where, however, the enzyme is more stable (19) and the effects of many added reagents are apparently accentuated. Most of our results are based upon work in the lower pH range, although comparisons were frequently made at pH 9.5.

*Activating Effects of Metal Ions*—The activating effect of ferrous ion upon arginase has already been emphasized by several authors. It has been shown that this activation is often more reliable and pronounced when the  $\text{Fe}^{++}$  is used together with organic thiol

TABLE I

*Effects of Reagents upon Arginase Activity, Activations, Inactivations, and Reactivations by Specified Reagents*

The procedure was essentially the same as for the *typical experiment* described under "Methods," exceptions are noted. Activities are expressed as ml of 0.02 N HCl (corrected), equivalent to  $\text{NH}_3$  present in 10 ml. of filtrate after urease action (10 ml.  $\approx$  two-fifths of the reaction mixture). Corrections were applied on the basis of controls (*cf.* "Methods").

Arginase solution*	0.02 N HCl corresponding to initial activity	Reagents added	0.02 N HCl corresponding to activity
	ml.		ml
A to F†	3.42	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	6.35
G	3.06	$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 2 mg.	5.56
Same		$\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 3 mg	5.61
H†	4.19	$\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 3 "	6.00
Same		$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 3 mg.	3.56
I§	5.02	Cysteine HCl (neutralized), 6 mg	4.31
Same		$\text{I}_2$ , 0.01 N, 0.6 ml.	0.00
"		Same + $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 3 mg	7.29
J	3.65	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 3 mg.	6.46
Same		$\text{NaHSO}_3$ , 5 mg	3.34
"		Benzoquinone, 1 mg	0.34
"		Same + $\text{NaHSO}_3$ , 5 mg.	0.94
"		" + $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 3 mg.	4.87
"		" + $\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 3 mg	3.73
"		" + $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 3 mg	0.28
"		" + cysteine HCl (neutralized), 6 mg.	1.71
"		" + " " + $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 3 mg.	3.94
K	3.60	$\text{Fe}_2(\text{SO}_4)_3$ , 2 mg	0.20
"		Same + $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 3 mg ¶	2.51
"		$\text{Fe}_2(\text{SO}_4)_3$ , 2 mg **	2.47
L	5.90	$\text{C}_6\text{H}_5\text{HgOH}$ , 3 mg. + $\text{C}_6\text{H}_5\text{CH}_2\text{HgCl}$ , 3 mg ††	5.79
M††	3.86	$\text{K}_3\text{Fe}(\text{CN})_6$ , 10 mg.	2.24
"		$\text{K}_3\text{Fe}(\text{CN})_6$ , 3.3 mg.	2.48
"		$\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$ , 4.2 mg	3.65
"		$\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 2 mg. + $\text{K}_3\text{Fe}(\text{CN})_6$ , 3.3 mg	5.23
"		$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 2 mg. + $\text{K}_3\text{Fe}(\text{CN})_6$ , 3.3 mg.	4.37
N§§	3.00	$\text{H}_2\text{S}$ water (saturated; about 0.1 M), 5 ml	0.75
Same		Same + $\text{N}_2$ treatment	1.85
"		" + " " + $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 3 mg.	6.89

\* Arginase solutions are arbitrarily lettered, for convenience

TABLE I—*Concluded*

† Average of six determinations; the enzyme solution in each instance was freshly prepared from the same stock sample of powdered arginase; initial activities varied, 3.12 to 3.67 ml.; with cobalt (0.1 to 3 mg.), 6.44 to 6.85 ml. Magnitude of corrections: determination with cobalt, enzyme omitted, 0.33 ml.; with cobalt, *boiled enzyme solution* present, 0.43 ml.

‡ Deviations from *typical determination*: arginase solution 1.0 ml., glycine buffer (0.02 M, pH 9.5) 10 ml., instead of phosphate in the initial mixture; cobalt or zinc salt added 15 minutes before the addition of buffer.

§ The initial reaction mixture to which reagents were added consisted of 5 ml. of arginase, 4 ml. of water, and 10 ml. of phosphate (1 M, pH 7.5); the volume of the arginase-arginine digestion mixture, 21 ml.; the reagent first added was permitted to act 30 minutes before further additions. The addition of cysteine with  $\text{Co}^{++}$  (after  $\text{I}_2$ ), in a parallel determination, introduced no observable change from the effect of  $\text{Co}^{++}$  alone; "activity" when  $\text{Co}^{++}$  was added directly to the initial mixture, 8.24 ml. (93 per cent hydrolysis); for  $\text{Ni}^{++}$  instead of  $\text{Co}^{++}$ , 7.77 ml. When urea, 10.0 mg., was added after iodine and  $\text{NaHSO}_3$ , 10 mg. (arginine present and arginase omitted), the results were: found, 6.53 ml.; calculated for 4.0 mg. of urea ( $\approx$  10 ml. of urease-filtrate), 6.67 ml. The magnitude of reactivation from iodine by nickel or manganous salts, or  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (added as a solid after the addition of some cysteine), was of the order of 60 to 75 per cent of the cobalt effect. Cysteine, alone, occasionally effected slight reactivation.

|| Each added reagent was permitted to act 20 minutes before any further addition. Control determinations of urea in the presence of arginine and various added reagents (arginase omitted) gave satisfactory results.

¶ Ferric salt added before phosphate; phosphate added before cobalt addition.

\*\* Added *after* phosphate.

†† Added to arginase, 5 ml.; the mixture stood 1 hour before the addition of 10 ml. of phosphate.

‡‡ Reagents were added (after phosphate) in the order named in intervals of 20 minutes; somewhat similar effects were observed when glycine (pH 9.5) buffer was used after addition of reagents to the enzyme, but the magnitude of the changes was less.

§§ The second and third digestions were in pure  $\text{N}_2$  (*cf.* "Other reagents").

compounds or certain other substances with which it might combine to form ferrous complex ions. Such results have also been obtained in this work. This action of ferrous ion is usually interpreted in terms of a reduction. It seemed to us that this interpretation might require modification in view of our observations of the failure of reducing agents of greater potency, such as  $\text{HCN}$ ,

H<sub>2</sub>S, organic thiol compounds, etc., to activate under varying conditions. Thus, the effect might be related to some other attribute of the ferrous ion, specifically the property of coordination (*cf.* (26)) with suitable molecules (*e.g.* derivatives of NH<sub>3</sub>). This conception, which involves no unique property of ferrous ion, prompted experimentation with a variety of ions, with the result that ions of the other metals of the iron triad (Co<sup>++</sup> and Ni<sup>++</sup>) and, in addition, Mn<sup>++</sup> (*cf.* (27)) were found to be excellent activators. Typical results are recorded in Table I. The arginase in freshly prepared enzyme powder is more active than in older preparations, but the enzyme in preparations several months old is extensively activated in the presence of these ions. The effects are less pronounced at pH 9.5 (glycine buffer) than at pH 7.5, and this is especially true for Ni<sup>++</sup>. Cobaltous ion appears to be the most potent activator. The ions, Cd<sup>++</sup> and Zn<sup>++</sup>, exhibit no comparable effect; Cd<sup>++</sup> possibly activates slightly, while Zn<sup>++</sup> is inert or actually depressant (*e.g.* at pH 9.5). Certain ions abolish the activity, apparently in part by precipitation of enzyme substance. Examples are Cu<sup>++</sup>, Hg<sup>++</sup>, Ag<sup>+</sup>, and PtCl<sub>6</sub><sup>-</sup>. Partial restoration from the action of Cu<sup>++</sup> is occasionally effected by removal of the cupric copper by means of the prolonged action of a zinc-platinum couple. In such instances, the further addition of Co<sup>++</sup> results in increased reactivation.

From controlled inactivations of arginase by quinone, iodine, or ferric ion, the activity is readily restored and usually maintained at its maximum by suitable addition of cobaltous ion. Ni<sup>++</sup> and Mn<sup>++</sup> act similarly but in different degree.

*Magnitude of Activation by Co<sup>++</sup>*—This is most conveniently gaged by a comparison of the relative rates of hydrolysis of arginine, catalyzed by arginase with and without Co<sup>++</sup>. Table II records some preliminary results. Such observations are being extended; their implications for arginase action need not be discussed here. It may be noted that, in the determinations *with cobalt*, the reaction constant, calculated on the basis of an uncomplicated pseudounimolecular reaction, exhibits over the range  $t = 31.5$  to 181.5 minutes (arginine, about 85 per cent hydrolyzed) a rather small deviation from the average value. This may be somewhat fortuitous with the few data presented, but is certainly less true for the hydrolysis *without cobalt*. The data afford a

basis for rough comparison, and show, further, that under the conditions the hydrolysis, *with cobalt*, is apparently nearer completion after 20 hours or less than after 48 hours, *without cobalt*.

These observations suggest possible advantages in the use of cobaltous chloride with arginase, under proper conditions, in certain estimations of arginine, *e g.* in biological material.

TABLE II

*Magnitude of Activation by Cobaltous Ion*

The procedure was similar to that of the *typical experiment* described under "Methods." The determination after 121.5 minutes was inadvertently omitted in the series *with cobalt*. Each result represents an independent determination; all determinations were started on the same day, one preparation of arginase solution serving as source of enzyme for all.

The reaction constants,  $K$  and  $K_{Co}$ , were calculated rather empirically with the use of the equation  $K = 1/t \ln(a/a - x)$ ; time,  $t$ , is expressed in minutes; for  $a$  is used 8.66, the number of ml (corrected) of 0.02  $N$   $HCl$  found equivalent to ammonia in 10 ml of urease-filtrate after 48 hours hydrolysis of arginine (cobalt present), as compared with 8.82, ml. *calculated* (complete hydrolysis of the arginine used); for  $x$  is used the corresponding number of ml (corrected) of 0.02  $N$   $HCl$  found equivalent to urea set free during time of arginine hydrolysis,  $t$ .

Time of hydrolysis	Arginase (non-activated)		Arginase + $CoCl_2 \cdot 6H_2O$	
	0.02 $N$ $HCl$ equivalent to urea formed	$K$ (calculated)	0.02 $N$ $HCl$ equivalent to urea formed	$K_{Co}$ (calculated)
<i>min</i>	<i>ml</i>		<i>ml</i>	
31.5	1.39	0.0056	2.43	0.0104
61.5	1.81	0.0038	4.01	0.0101
121.5	3.31	0.0040		
181.5	4.12	0.0036	7.45	0.0108
<i>hrs</i>				
20	7.10		8.67	
48	8.24		8.66	

*Other Characteristics of Activation by  $Co^{++}$* —Series of parallel determinations of arginase activity, influenced by  $CoCl_2 \cdot 6H_2O$  added to the reaction mixtures *seriatim* in specified increments from 0.001 mg. to 6.0 mg. show that the minimum concentration of  $Co^{++}$  for maximal activation *under the conditions* is of the order  $1.5 \times 10^{-5} M$ . This is illustrated by typical data of Table III. The corresponding optimal concentration for  $Ni^{++}$  may be placed at about  $2 \times 10^{-4} M$  (a preliminary value). The ions (without

arginase) do not as such display catalytic properties under the conditions; this is proved by controls and by series of experiments in which  $\text{Co}^{++}$  concentration is held above the optimal and the amounts of arginase varied, from which it appears that the catalytic action is directly proportional to the amount of arginase added, with or without  $\text{Co}^{++}$ ; the data are actually more consistent when  $\text{Co}^{++}$  is present.

TABLE III

*Activation of Arginase in Relation to Cobaltous Ion Concentration*

*Procedure*—The specified amount of cobalt chloride in 10 ml of water, added to arginase solution 0.5 ml., water 10 ml., and phosphate (1 M, pH 7.5) 5 ml.; the mixture stood 20 minutes before the addition of arginine carbonate 45 mg., in water, 10 ml.; digestion time, 3 hours;  $\text{NaHSO}_3$  (but not  $\text{NaCN}$ )\* omitted. The data are recorded in the two left-hand columns; initial activity, expressed as ml of 0.02 N HCl, 1.69. In the two right-hand columns are recorded data from a similar series, by the same procedure except that arginase solution, 2.0 ml., was used and the digestion time was 2 hours; initial activity, 3.97.

CoCl <sub>2</sub> 6H <sub>2</sub> O added	0.02 N HCl equivalent to urea formed	CoCl <sub>2</sub> 6H <sub>2</sub> O added	0.02 N HCl equivalent to urea formed
mg	ml	mg	ml
1.0	4.01	6.0	6.34
0.1	3.79	2.0	6.44
0.05	3.72	1.0	6.44
0.01	2.57	0.1	6.44
0.005	2.19	0.05	6.39
0.001	1.59	0.01	5.39

\* Parallel determinations: (1) arginase solution *boiled* before use (or omitted altogether), and  $\text{NaCN}$  omitted; found, 5.53 ml.; (2) the same, but arginine also omitted; found 0.21 ml. This illustrates the arginine-metal-urease phenomenon (*cf.* Table IV and "Discussion") and indispensability of  $\text{NaCN}$ .

*Inactivations by Oxidizing Agents; Reactivations by Certain Metal Ions*—Such oxidants as methylene blue and dipotassium indigo-disulfonate exhibited no observable effect upon the activity of arginase. Iodine or quinone drastically suppressed the activity. From *controlled* inactivations by these reagents the activity was, apparently, extensively recovered in the presence of  $\text{Co}^{++}$  especially and, perhaps, to a lesser degree, of  $\text{Ni}^{++}$  and  $\text{Mn}^{++}$  as well.

as  $\text{Fe}^{++}$ . The reactivating effect of  $\text{Fe}^{++}$  was dependable only when its use was preceded by that of some suitable reagent (*e.g.* cysteine). The other ions were effective when added alone. Reactivation from quinone was realized to a *limited degree* by the use of cysteine or bisulfites. Arginase activity was abolished by selenium dioxide (apparently irreversibly) and by ferric ion only when the addition to the enzyme of these reagents preceded the addition of phosphate; from inactivations by  $\text{Fe}^{+++}$ , the activity was readily restored by  $\text{Co}^{++}$ . The action of the system,  $\text{Fe}(\text{CN})_6^{=}: \text{Fe}(\text{CN})_6^{\equiv}$ , involved a noteworthy peculiarity. Ferrocyanide did not alter the enzyme activity significantly; depression resulted increasingly as the ratio of ferricyanide to ferrocyanide was increased in mixtures of these ions added to the enzyme; but complete inactivation of arginase was not readily effected, even by the use of the oxidant  $\text{Fe}(\text{CN})_6^{=}$  alone. When a sufficient amount of  $\text{Ni}^{++}$  or  $\text{Mn}^{++}$  was also present, the activity was not depressed. Table I shows typical results.

*Other Reagents*—Bisulfites and organic thiol compounds did not activate this arginase; cysteine depressed the activity somewhat. The supporting action of cysteine, when used with  $\text{Fe}^{++}$ , has been cited. It has been plausibly suggested (28) that such behavior is related to the maintenance in reaction mixtures of  $\text{Fe}^{++}$  in the reduced state. So potent a reductant as  $\text{TiCl}_3$  (in borate buffers) was found incapable of enhancing arginase activity under conditions where  $\text{Co}^{++}$  effected an increase. When these and other reagents were added to arginase, appropriate measures were taken to avoid injury to the enzyme by an inadvertent alkalization or acidification.

The action of HCN upon arginase at pH 7.5 was, in general, depressant (*cf.* (10)). At no time was activation by HCN observed.

Hydrogen sulfide markedly depressed arginase activity (at pH 7.5) *but partial restoration from the action of this reagent could be effected by simple removal of the excess* in a stream of air or nitrogen or by precipitation of sulfide ions with  $\text{Zn}^{++}$ . The further addition of  $\text{Co}^{++}$  ions established more extensive reactivation. Such results were also obtained when the reactions were carried out in an atmosphere of pure nitrogen. For this purpose, buffered arginase solution, alone or treated with  $\text{H}_2\text{S}$ , and arginine solution, with or without the addition of  $\text{Co}^{++}$ , were separately swept with nitrogen



(passed over CuO and Cu in the usual manner) in special all-glass apparatus, so constructed that the solutions could be mixed in the nitrogen atmosphere and the mixture maintained therein during the digestion and the subsequent destruction of the enzyme by heat. Arginase action can evidently take place in the absence of air and the *activations* observed are also, *apparently*, independent of an oxygen effect (*cf.* (15, 27, 28)).

The presence in reaction mixtures of mercaptide-forming organo-mercurials, such as phenylmercuric hydroxide and benzylmercuric chloride, had no observable effect upon the action of arginase (*cf.* (2)).

#### *Urease, Metals, and Arginase Activity*

From the inception of this work, we adopted the practice of adding sodium cyanide to the buffered reaction mixtures immediately before the addition of urease in the estimations of urea formed in the preceding step (hydrolysis of arginine). The primary purpose was to insure protection of the urease against a possible destructive action of some of the reagents (salts or organic derivatives of heavy metals) added earlier. For example, certain heavy metal ions would be bound as complex cyanide ions. Secondly, cyanide might serve to inactivate any residual traces of arginase. Further, the presence of cyanide is favorable to urease action (1).

In the later development of the work we were led by certain theoretical considerations to test, as possible catalysts for the hydrolysis of arginine (independent of arginase), mixtures of one of the ions,  $\text{Co}^{++}$ ,  $\text{Ni}^{++}$ ,  $\text{Mn}^{++}$ , with various synthetic nitrogen compounds that are known to coordinate with such ions. Under these conditions, the extensive hydrolysis of arginine was indeed observed, but a careful analysis of the situation soon disclosed that urease was in some manner involved. This was made evident by the following: (1) increase in the amount of cyanide added before the addition of urease or decrease in heavy metal ion concentration (and omission of any synthetic material) abolished the effect; (2) when arginase and NaCN were both omitted in the typical determination with  $\text{Co}^{++}$  (described in a preceding section), there was observed extensive hydrolysis of arginine. It is concluded that significant hydrolysis of arginine *in the absence of arginase* results

when urease and cobaltous ion are both present in the reaction mixture.

Subsequent investigation proved that  $\text{Co}^{++}$  may be replaced by  $\text{Ni}^{++}$ ,  $\text{Mn}^{++}$ , or (apparently)  $\text{Fe}^{++}$  plus cysteine. Indeed, with some samples of crystalline urease, particularly at a higher pH level, appreciable hydrolysis is observed without added metal. Does such urease "contain" effective metal (*e.g.*, as impurity) or does it function as such simply by the usual catalytic action upon urea hydrolysis, effecting the removal of one of the products of arginine hydrolysis? Further experimentation should clarify these and many other matters requiring explanation.

The urease-metal effect described was observed alike with crude jack bean urease and crystalline urease (29). Efforts to refer the effect to some constituent of jack bean other than urease have been unsuccessful.

Table IV records some of the results

Sodium cyanide, as applied in this investigation, apparently serves effectively to inhibit this urease-metal action. Its indispensability in the evaluation of arginase activity in this investigation needs no further comment. The bearing of these results upon analytical operations involving in general the use of urease in the determination of urea, where arginine and certain metal ions may also be present, is obvious.

### *Theoretical*

The experimental results emphasize certain noteworthy characteristics of arginase activation. Some of these are (1) the failure of several potent reducing agents, *e.g.* those that activate papain, to activate partially inactivated arginase, or to effect, under the conditions used, the extensive restoration of the activity after controlled inactivations by oxidants; (2) the competency of certain metal ions, *e.g.*  $\text{Co}^{++}$ ,  $\text{Ni}^{++}$ ,  $\text{Mn}^{++}$ , to effect such activations or reactivations; (3) the depressant or inactivating effect of  $\text{HCN}$  and  $\text{H}_2\text{S}$ , in contrast to the favorable action of these reagents upon papain or urease activity.

There are significant objections to an interpretation of the observed action of an ion like  $\text{Co}^{++}$  in terms of a reduction. In Table V are listed for comparison recorded values of the potentials of  $\text{Co}^{+++}:\text{Co}^{++}$  and other "positive" systems with those of several

less "positive" electromotively active systems. In so far as it is *permissible* to apply such data here it may be said that it is extremely unlikely that the reductant ion of so positive a system as  $\text{Co}^{+++}:\text{Co}^{++}$  might, of *itself*, function to reduce an enzyme. The

TABLE IV

*Hydrolysis of Arginine in Presence of Urease and Certain Heavy Metal Ions*

Crystalline urease prepared from 100 gm. of jack bean meal was dissolved in 25 ml. of water. Determinations were conducted as follows: metal salt was added to the mixture, urease solution 5 ml, water 10 ml., and phosphate buffer (1 M, pH 7.5) 3 ml.; after 20 minutes some caprylic alcohol and arginine carbonate, 45 mg., were added; digestion, 20 hours at 30°; 3 ml. of phosphate were added and the mixture held 10 minutes at 100°, and then brought to 25 ml;  $\text{NH}_3$  was determined in 10 ml. Results are expressed as ml. of 0.02 N HCl equivalent to  $\text{NH}_3$ .

Reagent added	0.02 N HCl
	ml
None.....	3.76*
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 2 mg	8.72††
$\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 3 mg	8.76
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 3 mg	8.67
Cysteine hydrochloride (neutralized) + $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 3 mg	6.83
$\text{C}_6\text{H}_5\text{HgOH}$ , 5 mg. + $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 2 mg.	0.19§

\* Parallel determinations: (1) crude urease (22), 0.1 gm, substituted for crystalline urease, HCl, 0.32 ml.; (2) digestion time (crystalline urease used), 2 hours (instead of 20 hours), HCl 0.17 ml.

† Controls: (1) boiled urease solution used and all other factors unchanged, HCl 0.10 ml.; (2) arginine (only) omitted, HCl 0.05 ml

‡ Parallel determinations: (1) crude urease, 0.1 gm., substituted for crystalline urease, HCl 8.82 ml.; (2) digestion time (crystalline urease used), 2 hours (instead of 20 hours), HCl 1.96 ml. Controls with crude urease: no  $\text{NH}_3$  obtained when only urease or arginine was omitted; nor when cobalt was present and urease or arginine was omitted

§  $\text{C}_6\text{H}_5\text{HgOH}$  inactivates urease (*cf.* (1)) but does not inhibit "true" arginase activity (*cf.* Table I); application of the xanthidrol test failed to detect urea in the final mixture.

limited degree of inactivation exhibited by ferricyanide ion is of interest in this connection. It has been shown that cobaltous ion reactivates arginase, initially treated with inhibiting ferric ion. This can scarcely be "reduction" in the accepted sense. Finally,

the failure of certain common reducing agents to reactivate has already been pointed out.

The depressant action of HCN and H<sub>2</sub>S upon arginase suggests that the enzyme, itself, may partake of the nature of a heavy metal complex (cf. (10)). If such a suggestion is tenable, there is provided a basis for the correlation of most of the facts observed. If the metallic component of such a complex molecule is present in a reduced state when the enzyme is active, the following might be assumed. Inactivation by oxidants might involve (1) simple oxidation of the complex, reversed by suitable reduction; or (2) separation of the metallic ion or component from the remainder of the molecule, possibly coincident with the oxidation of the former. The second, more drastic event, would correspond to usual experience. If so, suitable reduction might, under favorable circum-

TABLE V  
*Potentials\* of Various Oxidation-Reduction Systems*

System	Volts	System	Volt
Co <sup>+++</sup> Co <sup>++</sup>	1 81	Fe <sup>+++</sup> .Fe <sup>++</sup>	0 747
Ce <sup>++++</sup> Ce <sup>+++</sup>	1 5	$\frac{1}{2}$ I <sub>2</sub> I <sup>-</sup>	0 534
$\frac{1}{2}$ Cl <sub>2</sub> :Cl <sup>-</sup>	1 36	Fe(CN) <sub>6</sub> <sup>=</sup> .Fe(CN) <sub>6</sub> <sup>=</sup>	0 486

\* Washburn, E. W., International critical tables of numerical data, physics, chemistry and technology, New York, 6, 332 (1929).

stances, be accompanied by recombination of the enzyme fragments. However, *partial* reactivations are only occasionally brought about by reductants, while reactivation by Co<sup>++</sup>, etc., is more dependable and complete.

The depression of activity by hydrogen sulfide might conceivably result (a) by the formation of a loose addition compound with the metal-enzyme, from which the enzyme is regenerated by suitable removal of H<sub>2</sub>S; or (b) by disruption of the metal-enzyme and, possibly, conversion of the metallic component to a sulfide. It has been shown that a partial regeneration of activity is, indeed, readily obtained. It seems significant that, in this instance also, extensive restoration has been effected by the suitable addition of cobaltous ion.

A rather plausible explanation of the activating property of the

effective ions,  $\text{Co}^{++}$ ,  $\text{Ni}^{++}$ ,  $\text{Mn}^{++}$ , or  $\text{Fe}^{++}$ , rests upon the assumption that such ions (probably maintained in their reduced states) may replace, more or less readily, the metallic atom or component, characteristic of arginase, when that component has been lost to the enzyme by some process which has not injured the rest of the molecule. Such reactivation by "substitution" would be intimately related to the very characteristic property of these ions to form metal-coordinated complexes (*e.g.* the so called metal ammines). The metal ammines derived from reduced ions ( $\text{Me}^{++}$ ) are often less stable than their oxidized analogues. Their existence is, however, unquestioned. The assumed coordination in the activation picture might be conceived as involving both enzyme and substrate through contributions of suitable groupings by each (*e.g.* a portion of the guanido group of arginine). This assigns to the metal an interesting rôle in the building of an enzyme-substrate compound. It is conceivable that such coordination would eliminate resonance in the positively charged component (*i.e.* the guanidinium grouping) of the arginine zwitter ion. Molecular resonance has been plausibly assumed to exist (30) in the ion formed by addition of  $\text{H}^+$  to guanidine or a guanidino group. In the present instance, the "fixing" of bonds in the guanido group through enzyme-metal coordination might be reflected in promotion (acceleration) of hydrolysis in the direction of formation of ornithine and urea, with concomitant regeneration of the metal-enzyme. This hypothetical mechanism of arginase action may possibly find application in the further study of the arginine-metal-urease phenomenon already described.

#### SUMMARY

With the enzyme preparation used, it has been found that arginase, under the experimental conditions, is neither activated nor, after treatment with various oxidizing agents, reactivated by cysteine and other reducing agents that are known to activate papain. Arginase may, however, be effectively activated, or reactivated after treatment with oxidants, by certain metal ions, specifically  $\text{Co}^{++}$ ,  $\text{Ni}^{++}$ , or  $\text{Mn}^{++}$  (as well as  $\text{Fe}^{++}$ ). Cobaltous ion effects also the complete restoration of arginase activity after the enzyme has been inactivated by hydrogen sulfide and the excess of the latter removed.

Organomercurials of the type  $RHgX$  do not inactivate; ferri-cyanide ion suppresses the activity incompletely; and the ions,  $Cu^{++}$ ,  $Hg^{++}$ , and  $Ag^{+}$  destroy the activity, in part, by precipitation of the enzyme substance. The action of  $HCN$  is depressant.

The magnitude of activation by cobaltous ion is illustrated by appropriate rate measurements; certain other characteristics of the activations are also indicated

On the basis of the results, the arginase molecule is visualized as containing a metallic component which may be oxidized, or separated from the rest of the molecule by the action of oxidizing agents or certain other reagents. The observed actions of cobaltous ion and the other effective ions are interpreted, not as reductions, but rather in terms of the characteristic property of these ions to coordinate with suitable molecules or groupings to form complex molecules. This suggestion may include an implication regarding the rôle of the metallic component in the building of an enzyme-substrate compound.

Arginine, without arginase, is hydrolyzed in the presence of one of the ions,  $Co^{++}$ ,  $Mn^{++}$ ,  $Ni^{++}$ , and under certain conditions,  $Fe^{++}$ , when urease is also present in the reaction mixture. Organometallic suppressors of urease activity also suppress this effect. Cyanide diminishes the arginine-metal-urease effect, but, being a urease activator, does not prevent the independent hydrolysis of any urea also present in the reaction mixture. The bearing of these results upon the conduct of certain analytical operations is indicated.

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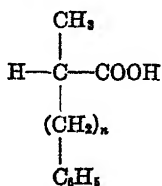
# CONFIGURATIONAL RELATIONSHIPS OF METHYLPHENYL- AND METHYLHEXYLACETIC ACIDS AND AN ATTEMPT AT THE CORRELATION OF THE CONFIGURATIONS OF 2-HYDROXY ACIDS WITH THOSE OF DISUBSTITUTED ACETIC ACIDS CONTAINING A METHYL GROUP .

By P. A. LEVENE AND STANTON A. HARRIS

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

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In the series of acids of the general type



( $n = 0$  or an integer) the configurational relationships of the acids with  $n = 1$  and 2 have already been established.<sup>1-3</sup> In order to elucidate the effect on the rotations of the acids of this type of the distance of the  $\text{C}_6\text{H}_5$  group from the asymmetric center, there remained to correlate the configuration of the acid with  $n = 0$  to the above two acids.

Inasmuch as the configurations of methylphenylacetic acid to methylethylcyclohexylmethane had already been established, there remained to correlate the configuration of this hydrocarbon to that of methylethylacetic acid. The principal set of reactions by which this task was accomplished is given in Table I.

From the fact that levo-methylethylcyclohexylmethane (IV) is correlated to dextro-methylethylacetic acid (I) and dextro-methylethylhexylmethane (VI), it follows that dextro-methylcyclohexylacetic acid (VII) is correlated to dextro-methylhexylacetic acid (II) and to levo-methylethylhexylmethane (III). It therefore

<sup>1</sup> Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **110**, 299, 311 (1935).

<sup>2</sup> Levene, P. A., *J. Biol. Chem.*, **110**, 323 (1935).

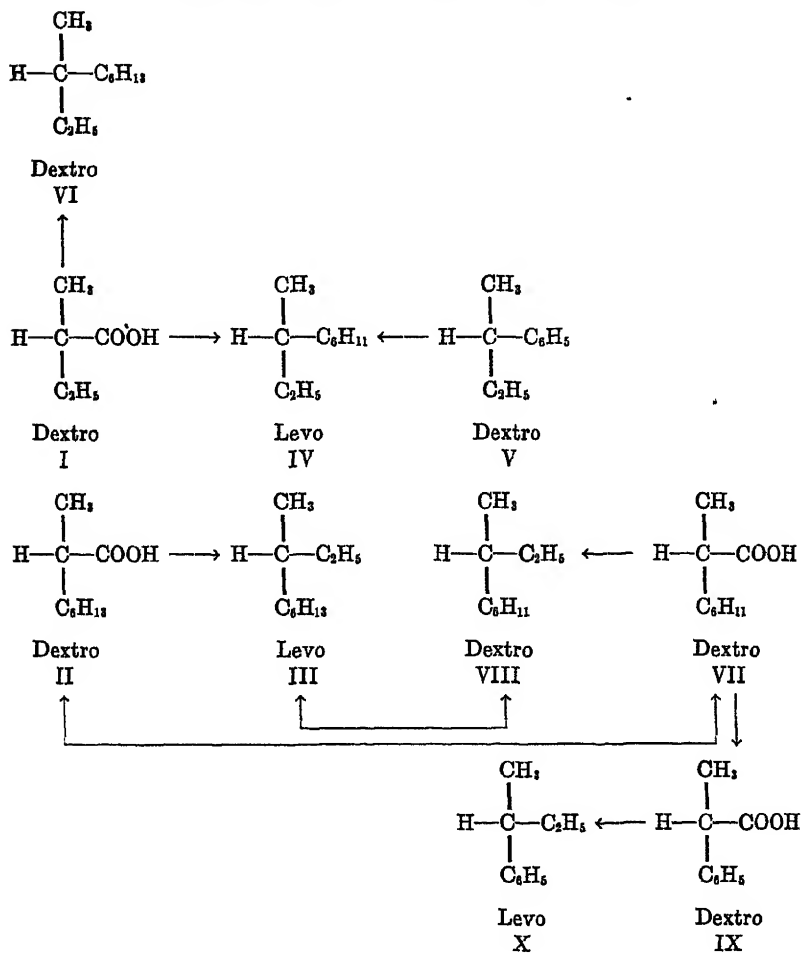
<sup>3</sup> Levene, P. A., and Harris, S. A., *J. Biol. Chem.*, **111**, 725 (1935).



follows on the basis of the observations of Levene and Marker,<sup>1</sup> of Levene,<sup>2</sup> and of Levene and Harris<sup>3</sup> that the configurations of

TABLE I

*Set of Reactions Leading to Correlation of Configuration of Derivatives Containing a Hexyl, Cyclohexyl, or Phenyl Group*





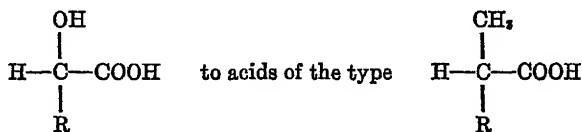
the acids containing a phenyl or a cyclohexyl group are correlated on the one hand among themselves and the other hand to the corresponding normal acids, as given in Table II.

*Effect of the Distance from the Asymmetric Center of Phenyl and Cyclohexyl Groups on Rotations of the Substituted Acetic Acids*

*Case of the Phenyl Group*—It may be seen from Table II that the substitution of the *n*-hexyl group by a phenyl enhances the rotation to a very high degree. In the case of the substitution of an *n*-heptyl group by a benzyl group, the rotation is only slightly enhanced. Thus the removal of the  $C_6H_5$  group at a distance of one  $CH_2$  group seems to have little effect on the rotation, but the substitution of the normal octyl group by a phenethyl again brings about an increase of the rotation in the same direction. Thus there seems to be an alternation in the shift of the rotation of the acids of the first row of Table II with the successive increase of the distance of  $C_6H_5$  from the asymmetric center.

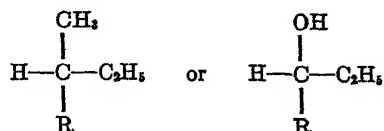
*Case of the Cyclohexyl Group*—In this case no marked difference is observed between the rotation of the normal and cyclic series. No periodicity is detected in either of the two series.

*Attempt to Correlate  $\alpha$ -Hydroxy Acids with Corresponding Normal Disubstituted Acetic Acids*—The question arises whether the knowledge of the configurations of the hydrocarbons given in Table II may serve for the correlation of acids of the type



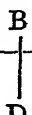
(R = a normal alkyl group, phenyl, benzyl, phenethyl, and their hydrogenated groups).

This task can be accomplished if it is admitted with Boys<sup>4</sup> that for simple substances such as

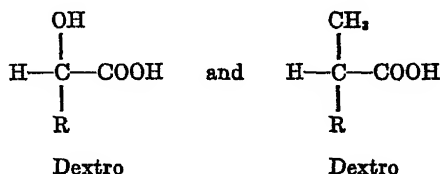


(R = C<sub>3</sub>H<sub>7</sub> or a higher homologous radicle) the absolute configuration is determined by clockwise or counter-clockwise arrangement of the radicles according to their increasing volume (H < OH < CH<sub>3</sub> < C<sub>2</sub>H<sub>5</sub> < C<sub>n</sub>H<sub>2n+1</sub> (n = 3 or a higher integer). This assumption was borne out by the experimental observations of Levene and Haller<sup>5</sup> and Levene and Marker.<sup>6</sup> Boys defines such spatial configurations as dextrorotatory which, viewed from the group with highest volume towards the observer, have the remaining three groups in clockwise order according to the descending volumes of the groups A < B < C < D.

In projection the dextrorotatory hydrocarbons may be represented in such a way that groups are arranged clockwise in descending order of volume, the groups A and C situated in the plane of the paper and the groups B and D in a plane above the paper. The

plane of the paper is represented by the lighter line as C —  A.

On the basis of this assumption the above hydrocarbon and carbinol are considered levorotatory. It would then follow that



(R = as above for both substances) are configurationally related.

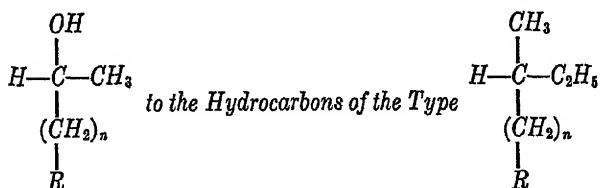
<sup>4</sup> Boys, S. F., *Proc. Roy. Soc. London, Series A*, **144**, 655 (1934).

<sup>5</sup> Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **79**, 475 (1923).

<sup>6</sup> Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 761 (1931).

TABLE III—Maximum Molecular Rotations of Configurationality

$\begin{array}{c} \text{OH} \\   \\ \text{H}-\text{C}-\text{C}_2\text{H}_5 \\   \\ \text{C}_6\text{H}_{13} \end{array}$	$\begin{array}{c} \text{OH} \\   \\ \text{H}-\text{C}-\text{C}_2\text{H}_5 \\   \\ \text{C}_6\text{H}_{11} \end{array}$	$\begin{array}{c} \text{OH} \\   \\ \text{H}-\text{C}-\text{C}_2\text{H}_5 \\   \\ \text{C}_6\text{H}_5 \end{array}$	$\begin{array}{c} \text{OH} \\   \\ \text{H}-\text{C}-\text{C}_2\text{H}_5 \\   \\ \text{CH}_2 \\   \\ \text{C}_6\text{H}_{13} \end{array}$	$\begin{array}{c} \text{OH} \\   \\ \text{H}-\text{C}-\text{C}_2\text{H}_5 \\   \\ \text{CH}_3 \\   \\ \text{C}_6\text{H}_{11} \end{array}$
-11 6°	+11 5°	+39 7°	-10 6°	-42.0°
$\begin{array}{c} \text{CH}_3 \\   \\ \text{H}-\text{C}-\text{C}_2\text{H}_5 \\   \\ \text{C}_6\text{H}_{13} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{H}-\text{C}-\text{C}_2\text{H}_5 \\   \\ \text{C}_6\text{H}_{11} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{H}-\text{C}-\text{C}_2\text{H}_5 \\   \\ \text{C}_6\text{H}_5 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{H}-\text{C}-\text{C}_2\text{H}_5 \\   \\ \text{CH}_2 \\   \\ \text{C}_6\text{H}_{13} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{H}-\text{C}-\text{C}_2\text{H}_5 \\   \\ \text{CH}_3 \\   \\ \text{C}_6\text{H}_{11} \end{array}$
-12 5°	+19 5°	-36 5°	-12 5°(App.)	-19 3°

*Correlation of Phenylated Secondary Carbinols of the Type*

In the above  $n = 0$  or an integer, R = a phenyl or cyclohexyl group.

Inasmuch as the carbinols of the above type with  $n = 0, 1$ , and 2 have been previously correlated among themselves and every one to the corresponding normal secondary carbinol,<sup>7</sup> all the carbinols given in the first row of Table III may be correlated to

<sup>7</sup> Levene, P. A., and Stevens, P. G., *J. Biol. Chem.*, 89, 471 (1930); 87, 375 (1930). Levene, P. A., and Walti, A., *J. Biol. Chem.*, 94, 367 (1931). Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, 97, 379 (1932).

*ated Secondary Carbinols and Trisubstituted Methanes*

$\begin{array}{c} \text{OH} \\   \\ \text{H}-\text{C}-\text{C}_2\text{H}_5 \\   \\ \text{CH}_2 \\   \\ \text{C}_6\text{H}_5 \end{array}$ <p>-51.7°</p>	$\begin{array}{c} \text{OH} \\   \\ \text{H}-\text{C}-\text{C}_2\text{H}_5 \\   \\ \text{CH}_2 \\   \\ \text{CH}_2 \\   \\ \text{C}_6\text{H}_{13} \end{array}$ <p>-10.7°</p>	$\begin{array}{c} \text{OH} \\   \\ \text{H}-\text{C}-\text{C}_2\text{H}_5 \\   \\ \text{CH}_2 \\   \\ \text{CH}_2 \\   \\ \text{C}_6\text{H}_{11} \end{array}$ <p>-10.0°</p>	$\begin{array}{c} \text{OH} \\   \\ \text{H}-\text{C}-\text{C}_2\text{H}_5 \\   \\ \text{CH}_2 \\   \\ \text{CH}_2 \\   \\ \text{C}_6\text{H}_5 \end{array}$ <p>-31.0°</p>
$\begin{array}{c} \text{CH}_3 \\   \\ \text{H}-\text{C}-\text{C}_2\text{H}_5 \\   \\ \text{CH}_2 \\   \\ \text{C}_6\text{H}_5 \end{array}$ <p>-9.25°</p>	$\begin{array}{c} \text{CH}_3 \\   \\ \text{H}-\text{C}-\text{C}_2\text{H}_5 \\   \\ \text{CH}_2 \\   \\ \text{CH}_2 \\   \\ \text{C}_6\text{H}_{13} \end{array}$ <p>-17.4°</p>	$\begin{array}{c} \text{CH}_3 \\   \\ \text{H}-\text{C}-\text{C}_2\text{H}_5 \\   \\ \text{CH}_2 \\   \\ \text{CH}_2 \\   \\ \text{C}_6\text{H}_{11} \end{array}$ <p>-16.8°</p>	$\begin{array}{c} \text{CH}_3 \\   \\ \text{H}-\text{C}-\text{C}_2\text{H}_5 \\   \\ \text{CH}_2 \\   \\ \text{CH}_2 \\   \\ \text{C}_6\text{H}_5 \end{array}$ <p>-35.10°</p>

the hydrocarbons of the above type as given in Table III.

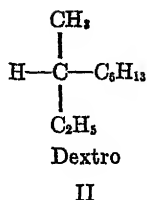
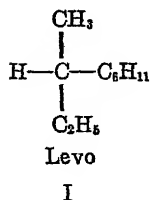
If the validity of the above conclusion is admitted, it is possible to speculate on the effect on rotation of substitution of a hexyl group by a phenyl or by a cyclohexyl in the case of the carbinols of the above type and of the corresponding hydrocarbons.

From Table III it may be seen that the substitution of  $\text{C}_6\text{H}_{13}$  by  $-\text{C}_6\text{H}_{11}$  when  $n = 0$  or 1 has a similar effect on the rotation in the case of the carbinols and in that of the hydrocarbons; the effect of substitution by  $\text{C}_6\text{H}_5$  is in the opposite sense in the case of the carbinols as compared with that of the hydrocarbons.

When  $n = 2$  the substitution of  $\text{C}_6\text{H}_{13}$  by  $\text{C}_6\text{H}_{11}$  has little effect on the molecular rotation of the substances; substitution by  $\text{C}_6\text{H}_5$  produces an identical effect in the case of the carbinol and in that of the hydrocarbon.

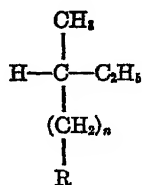
## SUMMARY

1. The similarity of the configurations of the hydrocarbons I and II

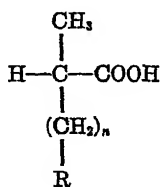


has been established by direct chemical methods

2 This correlation permitted (a) the correlation of the configurations of hydrocarbons of the general type

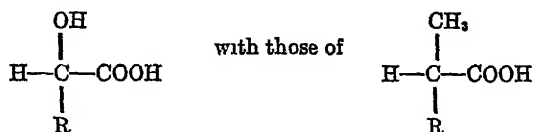


( $n = 0, 1, \text{ or } 2$ ;  $\text{R} = \text{C}_6\text{H}_{13}, \text{C}_6\text{H}_{11}, \text{ and } \text{C}_6\text{H}_5$ ), (b) the correlation of the configurations of acids of the general type



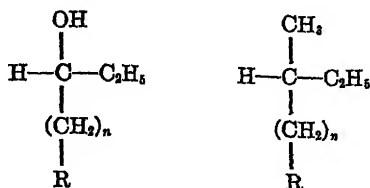
( $n$  and  $\text{R} = \text{as above}$ ), and thus to follow the effect on the rotation of the distance from the asymmetric carbon atom of the phenyl and cyclohexyl groups on the rotation of these acids

3. An attempt was made to correlate the configurations of acids of the general type



( $\text{R} = \text{a normal aliphatic radicle}$ ).

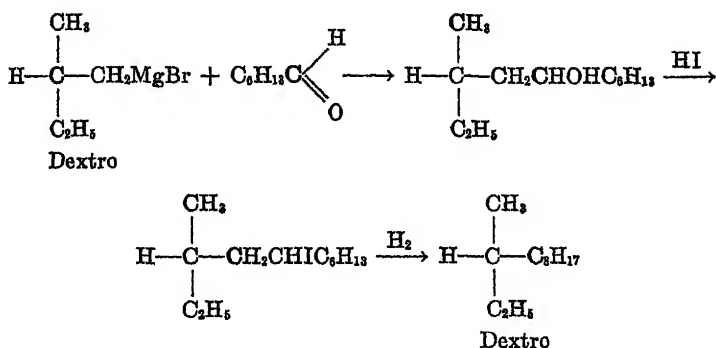
4. An attempt was made to correlate secondary carbinols and hydrocarbons of the types given below.



( $n = 0, 1, \text{ or } 2$ ;  $\text{R} = \text{C}_6\text{H}_{13}, \text{C}_6\text{H}_{11}, \text{ and } \text{C}_6\text{H}_5$ ).

*Synthesis of the Optically Active Methylethylcyclohexylmethane and Its Correlation to Methylethylcyclooctylmethane*—The configurations of the two hydrocarbons were correlated through their syntheses.

The *aliphatic hydrocarbon* was prepared by the following set of reactions.



The *methylethylcyclohexylmethane* was prepared in the following way. Grignard and Vignon<sup>3</sup> describe the preparation of 1-methylcyclohexanol-1 by adding ethyl acetate to pentamethylenedimagnesium bromide. Likewise it should be possible to obtain active 1-sec.-butylcyclohexanol-1 by treating the same Grignard reagent with the active ethyl ester of methylethylacetic acid. When the reaction was carried out, a carbinol having the correct composition was obtained, but on dehydration and reduction of the unsaturated compound an active hydrocarbon was obtained which did not have the correct physical properties as given by Signaigo

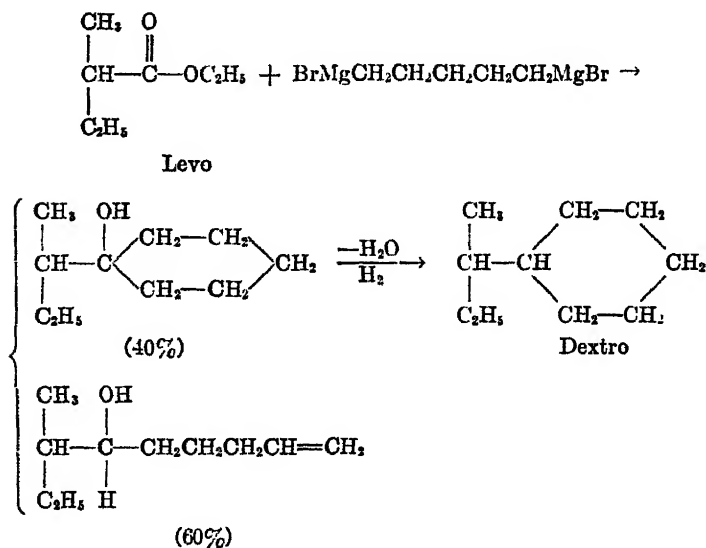
<sup>3</sup> Grignard, V., and Vignon, G., *Compt rend Acad.*, **144**, 1358 (1907).



and Cramer<sup>9</sup> and by Levene and Marker.<sup>10</sup> On examination of the physical properties of the carbinol and of the unsaturated hydrocarbon it was found that they did not correspond to those of homologous or isomeric compounds described by Signaigo and Cramer.<sup>9</sup>

It was then discovered that the reaction product contained a highly unsaturated compound. The product absorbed 60 per cent of the theoretical amount of bromine, calculated on the basis of the carbinol. On distillation the forerun spontaneously lost water and proved to be a mixture of active sec.-butylcyclohexene and active sec.-butylcyclohexanol. From it the active sec.-butylcyclohexane was prepared as described in the experimental part.

The high boiling dibromide mentioned above had a composition of a non-cyclic dibromo secondary carbinol  $C_{16}H_{20}OBr_2$ . The structure of the unsaturated carbinol and of its dibromide was not further investigated. The course of the reaction may be formulated in the following way.



<sup>9</sup> Signaigo, F. K., and Cramer, P. L., *J. Am. Chem. Soc.*, **55**, 3326 (1933).

<sup>10</sup> Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **97**, 563 (1932).

## EXPERIMENTAL

*Levo-1-Sec.-Butylcyclohexanol-1*—Pentamethylene bromide was prepared as described in "Organic syntheses,"<sup>11</sup> except that it was found necessary to shake a pentane solution of the bromide with cold concentrated sulfuric acid to remove all of the benzonitrile.

The reaction between this dibromide and magnesium in ether solutions was easily started by adding a few crystals of iodine 90 gm. (0.39 mole) of pentamethylene bromide in ether solution was added dropwise to an excess of magnesium (20 gm ; 0.83 mole) which was covered by ether. The total volume at the end of the reaction was about 500 cc. To this non-homogeneous Grignard reagent was added active ethyl ethylmethylacetate, having

$$[\alpha]_D^{25} = \frac{-6.18^\circ}{0.870} = -7.11^\circ$$

until the RMgX was completely destroyed. The ethyl ester was prepared in the usual manner from active ethylmethylacetic acid

$$[\alpha]_D^{20} = \frac{-7.81^\circ}{0.934} = -8.36^\circ$$

which was resolved as described by Schutz and Marckwald.<sup>12</sup>

The carbinol was obtained by first hydrolyzing the reaction mixture with aqueous ammonium chloride solution and then extracting with ether which was washed with water and dried over sodium sulfate. The carbinol boiled constantly at 99° at 14 to 16 mm. pressure. Yield 45 gm (0.288 mole) or 74 per cent of the theoretical.  $\alpha_D^{25} = +3.96^\circ$ .

This carbinol was treated in the following manner. 19.5 gm. (0.125 mole) of the carbinol were dissolved in chloroform and cooled in an ice bath. 20 gm. of bromine (0.125 mole) were dissolved in chloroform and made up roughly to a volume of 50 cc. This was added to the cooled carbinol solution until the red bromine color persisted. Exactly 30 cc, or 60 per cent, of the theoretical amount of bromine were used. This solution was shaken, first with sodium bisulfite solution, then water, and finally sodium bicarbonate solution. It was dried over sodium sulfate and distilled under reduced pressure through a short

<sup>11</sup> Gilman, H, Organic syntheses, New York, coll 1, 419 (1932)

<sup>12</sup> Schutz, O., and Marckwald, W., *Ber. chem. Ges.*, 29, 52 (1896).

column. After the chloroform was removed, the low boiling material from the bromide was separated by distillation at 5 mm. pressure, with a bath cooled with solid carbon dioxide for a condenser. This distillate was worked up for the cyclohexyl derivatives as described below

The high boiling fraction distilled at 161° at 5 mm. pressure. The analysis corresponded to the theoretical for  $C_{10}H_{20}OBr_2$ .

4.456 mg. substance: 6.275 mg  $CO_2$  and 2.500 mg.  $H_2O$

3.690 " " : 4.390 "  $AgBr$

$C_{10}H_{20}OBr_2$ . Calculated. C 38.0, H 6.35, Br 50.6

Found. " 38.4, " 6.27, " 50.6

*Levo-1-Sec.-Butylcyclohexene-1*—The low boiling distillate obtained in the previous experiment was wet and was also unsaturated, indicating spontaneous desaturation on distillation. Without attempting to isolate the carbinol, the material was distilled at atmospheric pressure. Water was given off above 140° and the hydrocarbon came over between 170–178°. The hydrocarbon was taken up in petroleum ether, dried with calcium sulfate, and redistilled. The substance was slightly colored and turbid, so it was redistilled from sodium. Yield 5 gm. B.p. 172–174° uncorrected.  $D_{20/20} = 0.829$  (*in vacuo*).  $n_D^{20} = 1.4590$ .

$$[\alpha]_D^{20} = \frac{-3.36^\circ}{0.829} = -4.06^\circ$$

3.780 mg substance: 12.060 mg.  $CO_2$  and 4.400 mg  $H_2O$

$C_{10}H_{18}$  Calculated. C 87.0, H 13.0

Found. " 87.0, " 13.0

*Dextro-2-Cyclohexylbutane*—The sec.-butylcyclohexene which had not been distilled from sodium was dissolved in 20 cc. of absolute alcohol and reduced with hydrogen and 0.1 gm. of  $PtO_2$  as a catalyst. Reduction was complete in 3 to 5 minutes. The solution was then diluted with 10 volumes of water, centrifuged to remove the catalyst, and extracted with petroleum ether. This was dried over calcium chloride and then distilled. After redistillation from sodium the following constants were obtained B.p. 176–178° at 760 mm.  $D_{20/20} = 0.815$  (*in vacuo*).  $n_D^{20} = 1.4460$ .

$$[\alpha]_D^{20} = \frac{+0.48^\circ}{0.815} = +0.59^\circ$$

3.870 mg. substance: 12.125 mg.  $CO_2$  and 4.970 mg  $H_2O$

$C_{10}H_{20}$ . Calculated C 85.7, H 14.3

Found. " 85.4, " 14.4

*3-Methylundecanol-5*—26 gm. of active amyl bromide,  $\alpha_D^{25} = +2.40^\circ$  (homogeneous), were added to 5.5 gm. of magnesium turnings in 100 cc. of dry ether. After all the bromide was added, the mixture was refluxed for 10 minutes. Then 21 gm. of freshly distilled heptaldehyde dissolved in 35 cc. of dry ether were added without cooling. The reaction mixture was poured onto ice, ammonium chloride solution added, and the ether layer separated. The extracts were washed with ammonium chloride solution, water, and dilute carbonate, and then dried over anhydrous potassium carbonate, and distilled. B.p.  $70-75^\circ$  at 0.1 mm. pressure; also  $113^\circ$  at 12 mm pressure. Yield 14 gm.  $D_{24/4} = 0.8272$  (*in vacuo*).  $n_D^{25} = 1.4367$ .

The rotation of the substance was

$$[\alpha]_D^{25} = \frac{+2.93^\circ}{1 \times 0.827} = +3.54^\circ, [M]_D^{25} = +6.59^\circ \text{ (homogeneous)}$$

Maximum  $[M]_D^{25} = +17.4^\circ$  (homogeneous)

3 040 mg. substance · 8 625 mg.  $\text{CO}_2$  and 3 820 mg.  $\text{H}_2\text{O}$

$\text{C}_{12}\text{H}_{24}\text{O}$  Calculated. C 77.3, H 14.1

186.2 Found. " 77.4, " 14.1

*3-Methyl-5-Iodoundecane*—11 gm. of 3-methylundecanol-5,  $[\alpha]_D^{25} = +3.54^\circ$  (homogeneous), were placed in a bomb tube and cooled in a dry ice-acetone bath, and about 30 cc. of anhydrous hydrogen iodide were distilled into it. The tube was sealed and let stand at room temperature for 4 days. It was again cooled and opened. It was then allowed to come to room temperature overnight and then let stand for an additional 3 days in order to allow the excess hydrogen iodide to evaporate. The iodide was extracted with pentane, and the extracts were washed with dilute carbonate and water, and dried over calcium chloride and distilled. B.p.  $88^\circ$  at 1 mm. pressure. Yield 15 gm.  $D_{25/4} = 1.1972$  (*in vacuo*).

$$[\alpha]_D^{25} = \frac{+7.00^\circ}{1 \times 1.197} = +5.85^\circ; [M]_D^{25} = +17.3^\circ \text{ (homogeneous)}$$

Maximum  $[M]_D^{25} = +45.7^\circ$  (homogeneous)

3 620 mg. substance: 6 470 mg.  $\text{CO}_2$  and 2 760 mg.  $\text{H}_2\text{O}$

$\text{C}_{12}\text{H}_{24}\text{I}$ . Calculated. C 48.6, H 5.5

296.1 Found. " 48.7, " 5.5

*Methylethyl-n-Octylmethane*—15 gm. of 3-methyl-5-iodoundecane,  $[\alpha]_D^{25} = +5.85^\circ$  (homogeneous), were reduced with Raney's

catalyst in methyl alcohol and sodium hydroxide solution. This was shaken in an atmosphere of hydrogen for 48 hours. The hydrocarbon was extracted with pentane, and the extracts were washed with water and concentrated calcium chloride solution and dried over metallic sodium. The product was then distilled B p.  $94^{\circ}$  at 15 mm. pressure. Yield 7 gm.  $D_{25/4} = 0.7491$  (*in vacuo*).  $n_D^{25} = 1.4216$ .

$$[\alpha]_D^{25} = \frac{+2.90^{\circ}}{1 \times 0.749} = +3.87^{\circ}; [\text{M}]_D^{25} = +6.59^{\circ} \text{ (homogeneous)}$$

$$\text{Maximum } [\text{M}]_D^{25} = +17.4^{\circ} \text{ (homogeneous)}$$

4.410 mg substance · 13.690 mg.  $\text{CO}_2$  and 6.100 mg.  $\text{H}_2\text{O}$

$\text{C}_{12}\text{H}_{26}$	Calculated	C 84.6, H 15.4
170.2	Found.	" 84.7, " 15.5

## SOME REACTIONS OF AMMONOLYZED PARATHYROID HORMONE

By RICHARD G. ROBERTS, WILBUR R. TWEEDY, AND GEORGE H. SMULLEN

*(From the Departments of Physiological Chemistry, Chicago Medical School, and Loyola University School of Medicine, Chicago)*

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The first workers to show that the chemical properties of proteins are altered by treatment with liquid ammonia were McChesney and Miller (1), although Taft (2) had previously made a study of the solubility of certain proteins in liquid ammonia. Gebauer-Fuelnegg (3) found that the anaphylactic properties of edestin were altered by treatment with liquid ammonia. Miller and Roberts (4) found that proteins and certain related substances are acidic in liquid ammonia and liberate hydrogen when sodium is added to them in that medium, and that characteristic curves for each protein can be plotted by correlating the hydrogen evolved with the amount of sodium added. Roberts and Miller (5) showed that certain proteins, such as hemoglobin, containing known prosthetic groups such as hematin, exhibit exceptional catalytic action for the formation of hydrogen and sodium amide by the reaction of sodium on liquid ammonia. Tweedy (6) observed that parathyroid hormone activity was retained in a preparation kept in liquid ammonia in a sealed tube for 50 hours at 0–22°. Recently Tweedy, Bell, and Vicens-Rios (7) found that the activity of their parathyroid hormone preparation, in respect to its power of mobilizing calcium into the blood, was not altered by reduction either with catalytic hydrogen under pressure, or with sodium amalgam.

Since a solution of sodium in liquid ammonia produces a solvated electron which is one of the most powerful reducing agents known, the present work was undertaken in an attempt to reduce parathyroid hormone by adding varying amounts of sodium to it

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in liquid ammonia. In addition any catalytic action has been noted, and a study has been made of the hydrogen evolved and the variations in the activity of the hormone. An attempt has been made to correlate the activity, reduction, and acidic hydrogen with postulated active groups in the hormone, and with protein structure.

### EXPERIMENTAL

The liquid ammonia was dried over sodium by the method of Fernelius and Johnson (8).

The parathyroid hormone was prepared by Tweedy's method (9), and was standardized by the procedure of Collip and Clark (10). Thirty different dogs, 10 to 26 kilos in weight, were used. A dose of 3.5 mg. per kilo of body weight produced an average increase in serum calcium of 5 mg. per 100 cc. within 15 hours. The hormone preparation had been dried in a vacuum desiccator over calcium chloride for 3 months, but before treatment with liquid ammonia it was dried in a vacuum desiccator over concentrated sulfuric acid to constant weight. We then considered the preparation to be free from water. Several drying controls were run with different proteins and it was found that drying in an electric oven at 85° for as long a time as 48 hours, followed by drying in a vacuum over sulfuric acid for 48 hours, did not give a lower figure for the hydrogen evolved than that obtained by the method of drying described. Above 115° the protein controls began to darken.

If the parathyroid hormone preparation contains bound water or water of constitution of any kind, it most probably cannot be removed without inactivation of the hormone. While a small amount of bound water would give a slightly high figure for the total acidic hydrogen evolved, it would not alter the shape of the curves we have obtained, but would elevate each curve slightly on the ordinate axis.

A description of the apparatus has been given in a previous article (4).

0.5 gm. samples of the parathyroid hormone preparation and 200 cc. of liquid ammonia were used for each determination. The sodium added varied from 41.7 to 249.9 mg., and the hydrogen evolved from 2.19 to 53.0 cc. These quantities are calculated to

gm. equivalents in Fig. 1. The time of reaction varied from 90 seconds to 3 hours. Additional time was allowed for the hydrogen to be boiled out of the reaction flask. The reaction flask was mounted in a loose fitting, padded container so that it could be shaken vigorously. After the reaction was completed, the reaction flask was disconnected from the gasometer, and attached to a mercury seal, which permitted the excess liquid ammonia to boil

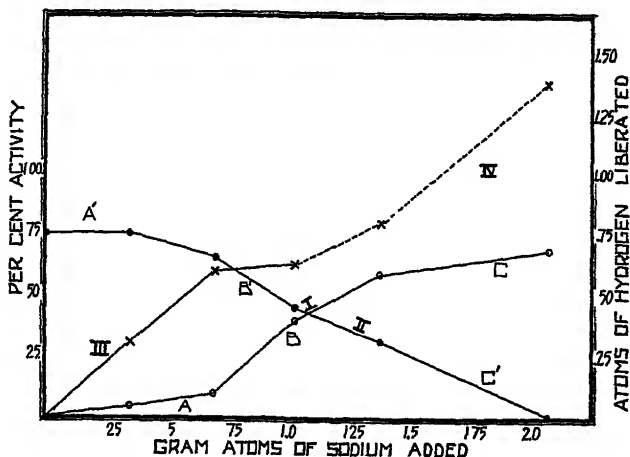


FIG. 1. For 1 gm. atom of nitrogen Curve I, gm. atoms of hydrogen evolved; Curve II, per cent of activity of hormone; Curve III, gm. atoms of sodium absorbed; Curve IV, gm. atoms of sodium absorbed plus excess available for absorption. The ordinate on the left should be used for Curve II only, and the ordinate on the right for Curves I, III, and IV. The letters A, B, and C and their primes found on Curves I and II represent segments of related activity.

off without the introduction of air or moisture into the reaction flask. This is important, since parathyroid hormone is inactivated when allowed to stand in an alkaline medium exposed to air. Dry liquid ammonia is not alkaline.

After the liquid ammonia had boiled off, a calculated amount of standard hydrochloric acid solution, sufficient to bring the reaction product to pH 6, was added to the Dewar flask. In adjusting the reaction product to pH 6, a portion of the sodium would be replaced by hydrogen during the acid hydrolysis. The



parathyroid hormone solution was then injected subcutaneously into dogs, and the blood serum calcium in duplicate samples was determined by the Kramer-Tisdall method as modified by Tweedy and Koch (11).

#### DISCUSSION

In Fig. 1 four curves have been drawn for the purpose of correlating the acidic hydrogen evolved with the change in hormone activity and the amount of reduction occurring to a given quantity of parathyroid hormone preparation, when varying amounts of sodium are added to it in liquid ammonia. Each of the first five points on the potency curve represents the average increase in serum calcium obtained in a different group of dogs, seven to eight in number, injected with a 3.5 mg. per kilo dose of the treated hormone preparation. The sixth point represents the average serum calcium increment obtained in a group of five dogs, which received a dose of 5.2 mg. per kilo. The quantity of parathyroid hormone preparation chosen for convenience in plotting is one atomic weight of Kjeldahl nitrogen, or 95.24 gm. The abscissa is expressed in gm. atoms of sodium added, and is to be used for all four curves. The ordinate on the left shows the per cent of hormone activity retained, expressing the potency of the untreated hormone preparation as 100 per cent. This ordinate is to be used only for Curve II. The ordinate on the right expresses the gm. atoms of hydrogen evolved, and is to be used directly only for Curve I. Indirectly, it can be used for Curves III and IV. Curve III represents the amount of sodium absorbed or decolorized, and is the total amount of sodium added minus the acid hydrogen evolved which is due to salt formation. Curve IV represents the amount of reduction occurring to the parathyroid hormone preparation, and is expressed numerically by the ordinate on the right, since 1 equivalent of sodium is equal to 1 equivalent of hydrogen. Curve IV does not represent the amount of reduction directly since the solution remained blue at this concentration, indicating an excess of sodium present; rather it indicates the amount of sodium available for reduction, which can be read in terms of hydrogen equivalents on the ordinate at the right.

It is seen that Curve II originates at 75 per cent activity instead

of 100 per cent The discrepancies of the method of bioassay may account for some of the apparent loss in hormone activity after brief contact with liquid ammonia. However, some of the loss in activity may be due to the action of liquid ammonia alone or to ammonolysis, and not to reduction or liberation of hydrogen. In previous publications (12, 13), Roberts has discussed the ammonolysis of other organic compounds.

In general Curves I and II resemble each other markedly, except that they are of an inverse order; as the acidity of Curve I increases the activity of Curve II decreases Curve I is typical of curves that are given by certain proteins when they are treated with varying amounts of sodium in liquid ammonia, and the quantities of sodium added are plotted against the hydrogen evolved. These protein curves have been discussed by Miller and Roberts (4). In the reaction described above parathyroid hormone reacts as a typical protein.

The shapes of Curves I and II indicate that the activity of parathyroid hormone is a direct function of a part of the acidic hydrogen in it. That no such relationship exists between the amount of reduction and the activity can be shown by comparing Curves II and III. When Curve III is rising most sharply, indicating very rapid reduction, the activity changes very little as shown by Curve II, and when Curve III flattens out showing practically no reduction, Curve II is changing most rapidly.

#### SUMMARY

1. Parathyroid hormone has no prosthetic group either with or without iron that has a catalytic effect for the action of sodium on liquid ammonia, such as that shown by hematin in hemoglobin.

2. The activity of parathyroid hormone is not dependent on disulfide linkages or on any linkages that are strongly reduced by sodium in liquid ammonia <sup>1</sup>

3. Parathyroid hormone reacts as a typical protein with sodium in liquid ammonia.

4. The activity of parathyroid hormone is lessened by the action of liquid ammonia alone.

5. It appears that the activity of parathyroid hormone is a

<sup>1</sup> Du Vigneaud and coworkers were able to reduce cystine by sodium in liquid ammonia (14)

function of a part of the acidic hydrogen liberated, and varies inversely with the liberation of this hydrogen, which most probably comes from imide groups as indicated by its slow evolution.

The authors are glad to record their indebtedness to the Committee on Scientific Research, American Medical Association (Grant 328),<sup>2</sup> for financial aid in the purchase of supplies, and technical assistance in the preparation of the hormone. They also wish to thank Swift and Company for their cooperation in supplying properly prepared glands.

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<sup>2</sup> Awarded to W. R. Tweedy.

# CHEMICAL STUDIES ON THE PITUITARY GONADOTROPIC HORMONE

BY L. C. MAXWELL AND FRITZ BISCHOFF

(From the Chemical Laboratory, Santa Barbara Cottage Hospital,  
Santa Barbara)

(Received for publication, August 23, 1935)

Fevold *et al.* (1) and Wallen-Lawrence (2) have reported the separation of a follicle-stimulating principle and a luteinizing principle from hypophyseal gonadotropic preparations. The latter author includes evidence for a chemical separation involving destruction of the luteinizing principle by formalin. Maxwell (3) on the other hand was able to obtain a pure follicular ovarian response simply by sufficiently distributing the dosage of unfracti-  
onated hypophyseal preparations.

In the present paper the formalin-treated hypophyseal preparations of Wallen-Lawrence were studied under various conditions of dosage. The action of various other chemically reactive compounds upon gonadotropic preparations was also investigated. It was hoped by this attack that evidence for or against the dual hormone theory and information relating to the structural groups concerned with the physiological activity might be obtained.

## EXPERIMENTAL

*Methods*—Since the chemical procedures in most instances are identical with those used by various workers in similar investigations on insulin or parathyroid preparations, the detailed procedure whenever possible has been omitted and reference made to the method employed.

Freshly prepared aqueous extracts (8 mg. per cc.) of a desiccated sheep hypophyseal preparation which corresponded to the Powder A of Wallen-Lawrence (2) with the pH 8.5 and the 40 per cent ethanol-insoluble proteins removed, were employed as the starting material. Unless otherwise stated all experiments were carried out at room temperature and in the presence of an excess of the

reagent. Control preparations were subjected to identical experimental conditions with each experiment. At the conclusion of the reaction the preparations were adjusted to pH 6.0, the protein fraction containing the active principle precipitated with 85 per cent ethanol, and taken up in aqueous solution or suspension for immediate assay. All experiments were made in duplicate or further repeated until consistent results were obtained.

*Assay*—21 to 23 day old female albino rats were dosed subcutaneously once daily for 4 days and sacrificed on the 6th day. With groups of eight animals, daily dosage equivalent to 1 mg., 2 mg., and 4 mg. of the powder used as the starting material gave ovaries weighing  $24 \pm 3$  mg.,  $45 \pm 10$  mg., and  $67 \pm 15$  mg. respectively. Due to the large individual variation in ovarian weight, it is obvious that only a semiquantitative evaluation of potency can be obtained without the use of large groups. Complete inactivation of the chemically treated preparations was considered to be accomplished when a total dose equivalent to 32 mg. of the original preparation failed to produce evidence of morphological changes in the ovaries or uterus on all of a group of three animals. As a roughly comparative evaluation of potency after chemical treatment, groups of three animals were dosed at each of the three dosage levels given above for the standard preparation.

*Formaldehyde*—The hypophyseal preparation was treated with varying concentrations of formaldehyde for periods up to 4 hours, the pH being maintained between 7 and 8. At the conclusion of the experiment excess formaldehyde was removed under a vacuum and the remainder destroyed by an excess of ammonia. The reaction was then adjusted to pH 6.0 and the gonadotropic fraction thrown out with 85 per cent ethanol. Typical experiments are presented in Table I. Although a marked loss in potency is observed, it will be noted that following prolonged treatment with formaldehyde a follicular ovarian response is obtained in the majority of instances when the increase in ovarian weight does not exceed 100 per cent.

Distribution of the dosage of formalin-treated preparations was investigated to determine whether the action of formalin could be attributed simply to a partial destruction of potency accompanied by a denaturation sufficient to retard absorption of the active

principle in the tissues. The experimental data (Table I) indicate that the small amount of active principle remaining in the formalin-treated preparation was so slowly absorbed in the tissues that the distribution of the dosage used was insufficient to alter the response significantly.

In order to determine whether a selective destruction of a luteinizing principle occurs, the action of formaldehyde upon a urine of pregnancy preparation was investigated 250 rat units of antuitrin S (Parke, Davis and Company) in 12 cc. of 6 per cent formal-

TABLE I  
*Effect of Formalin upon Ovarian Response to Gonadotropic Preparations*

Preparation	No of animals	Times dosed daily	Total dosage	Average ovarian weight*	No of luteal responses	Vagina open 9th day
Hypophyseal, untreated	8	1	4 mg.	24±3	7	1
	6	3	4 "	52±10	6	5
	8	1	8 "	45±10	8	5
	8	1	16 "	67±15	8	8
" 4% formalin 15 min.	3	1	16 "	86	3	3
" 10% " 4 hrs	6	1	16 "	20±3	2	6
	6	3	16 "	28±4	2	6
" 6% " 4 "	3	1	8 "	22	0	3
	3	3	8 "	24	0	3
Urine of pregnancy, untreated	5	1	10 rat units	35±7	5	5
" " " 6% formalin, 4 hrs.	5	1	10 " "	20±4	5	5

\* The mean plus the standard deviation (not the standard deviation of the mean) is given The weight is measured in mg

dehyde were allowed to stand at room temperature at pH 7 to 8 for 4 hours. Although some loss in potency occurred, no variation in the qualitative ovarian response from that of controls dosed with the untreated preparation could be demonstrated by microscopic examination of the sectioned ovaries. Assuming the same groups to be responsible for the luteinizing properties of both pituitary and pregnancy urine preparations, the results (Table I) fail to indicate a selective destruction of a luteinizing principle by formalin.

*Nitrous Acid*—Exposure of the hypophyseal preparation to an

excess of  $\text{NaNO}_2$  in 30 per cent acetic acid at  $37^\circ$  for 3 hours resulted in complete inactivation. A control preparation exposed to the same acidity also showed a very considerable loss in potency. When the experiment was repeated at  $10^\circ$ , no apparent loss in potency was observed in the control, while the physiological response both quantitatively and qualitatively of the treated preparation was practically identical to that reported for the preparations exposed to 10 per cent formaldehyde. At high dosage levels luteinized ovaries were obtained. On two series of three animals single daily dosage equivalent to 2 mg. of a preparation exposed to  $\text{HNO}_2$  at pH 4.0 for 1.5 hours at  $37^\circ$  produced a follicular ovarian response accompanied by uterine hyperemia and vaginal canalization, without causing a significant increase in the ovarian weight.

*Substitution and Addition Reactions*—Hypophyseal gonadotropic preparations were exposed to a variety of reagents known to react with amino, imino, or hydroxyl groups.

Rapid and complete inactivation occurred in alkaline solution (pH 8.0) in the presence of a moderate excess of the following reacting substances:  $\beta$ -naphthaquinonesulfonic acid, 15 minutes in the dark at room temperature; phenyl isocyanate, exposure for 1 hour at  $0^\circ$  (4); diazobenzenesulfonic acid (30 minutes,  $0^\circ$ ) (5); diazonitrobenzene (30 minutes,  $0^\circ$ ) (5); benzoyl chloride (30 minutes, room temperature) (5); carbon disulfide (1 hour in 60 per cent alcohol).

A 50 per cent to a 75 per cent destruction of the gonadotropic principle occurred without alteration of the qualitative nature of the response when hypophyseal preparations were exposed to the following reacting substances: benzoyl chloride (30 minutes, room temperature, pH 5.0); dimethyl sulfate (30 minutes, room temperature, pH 8.0); acetic anhydride (1 hour, room temperature)

Attempts to demonstrate reactivation of the acetylated and the methylated preparations by hydrolysis with N/30 NaOH at  $10^\circ$  were unsuccessful.

*Oxidizing and Reducing Agents*—Since inactivation with some of the foregoing reagents may have been due to oxidation or reduction, the effects of various oxidizing and reducing agents were investigated. The potency was found to be almost completely destroyed by 3 per cent  $\text{H}_2\text{O}_2$  in the presence of  $\text{NaHCO}_3$ . The

addition of a 10 per cent excess of iodine (0.01 N solution) over the amount found to combine in 1 hour with the proteins present was sufficient to destroy approximately 50 per cent of the activity. One of the iodine-treated preparations at minimal dosage levels gave follicular ovaries and an estrogenic response similar to the preparations treated with formalin. The addition of one-half the amount of iodine necessary to combine with the proteins was, however, without apparent effect upon either the quantitative or the qualitative nature of the response.  $\text{FeCl}_3$ , when incompletely removed previous to dosage, gave an augmented ovarian response (3).

No detectable loss of activity occurred upon exposure to nascent hydrogen, sulfur dioxide, hydrogen sulfide, ferrous sulfate, or hydrogen cyanide. Interest is attached to the qualitative nature of the assay of a preparation exposed to hydrogen sulfide, and also to one preparation treated with ammonium polysulfide. Both of these preparations gave strong estrogenic reactions at minimal dosage levels and an occasional follicular ovarian response.

*Acid and Alkali*—From 30 to 50 per cent of the potency of the hypophyseal preparation was lost by standing 3 hours at  $37^\circ$  in 0.1 N HCl. Complete inactivation resulted when the dry powder was dissolved in concentrated  $\text{H}_2\text{SO}_4$  at  $0^\circ$  according to the method employed by Bischoff and Sahyun (6) with insulin. Exposure for 20 hours at room temperature to 0.73 N HCl in 75 per cent ethanol also resulted in complete inactivation. Attempts to demonstrate reactivation of this preparation by hydrolysis with 0.08 N NaOH in the cold were unsuccessful.

Complete inactivation resulted with 0.1 N NaOH in 3 hours at  $37^\circ$ , while N/30 NaOH under these conditions destroyed from 30 to 50 per cent of the potency. Preparations exposed to N/30 NaOH at  $10^\circ$  showed no appreciable loss in potency. No change in the qualitative nature of the ovarian response was observed following exposure to either acid or alkali.

*Fate of Luteinizing Fraction*—Fevold *et al* (1) state that the luteinizing fraction when administered alone, even in excessive dosage, produces no marked increase in the ovarian weight. However, when dosage with the luteinizing fraction and the follicle-stimulating fraction are combined, an augmentation in ovarian weight is observed. The possibility that in our chemically in-



activated preparations a selective destruction of the follicle-stimulating principle had taken place was tested out experimentally. The results were negative. Combined dosage of the inactivated preparations with dosage of various preparations giving a follicular response failed to demonstrate an augmented ovarian response. In these experiments, which are not tabulated, low dosage with hypophyseal extracts containing  $\text{FeCl}_3$  or tannic acid (3), or extracts treated with formalin, was used to promote follicle stimulation.

#### DISCUSSION

If the activity of the more highly purified prolan preparations may be used as a basis of comparison, the purest gonadotropic pituitary preparations would appear to be relatively crude. In view of this question and the lack of precise methods of assay, the value of a chemical investigation of the nature described in this paper may be questioned. Results of similar early studies on non-crystalline insulin (5, 6) have to date not been greatly amplified by work on the crystalline material (7, 8) and were hence an incentive to this study. The present chemical studies appear informative and of value inasmuch as they establish a stability range to various chemical environments, which will tend to define the conditions for further work on the isolation of the gonadotropic principle or principles.

Much useless discussion has arisen in the insulin work as to the exact nature of the groups attacked by the various reagents. None of the reagents studied is exactly specific for any single chemical group. The present studies which are analogous indicate the amino, imino, or hydroxyl groups or their sulfur analogues may be concerned with the physiological activity of hypophyseal gonadotropic preparations.

The most significant finding appears to be that denaturation of the protein aggregate may produce the same physiologic effect as slowed tissue resorption of the original product. The evidence is therefore against the dual hormone theory.

#### SUMMARY

Reagents known to react with the amino, imino, or hydroxyl groups cause a partial to a complete inactivation of hypophyseal gonadotropic preparations.

The activity is completely destroyed by strong oxidizing agents but is unaffected by mild oxidizing agents or by reducing agents.

Complete inactivation occurs in 0.1 N NaOH, and partial inactivation in N/30 NaOH in 3 hours at 37°. 0.1 N HCl in 3 hours at 37° causes partial inactivation.

Evidence has not been obtained to support the dual hormone theory of gonadotropic preparations. The formalin-treated gonadotropic pituitary preparations show no augmentation effect when given in divided doses. The nature of the ovarian response to the urine of pregnancy preparation is not changed when formalin-treated preparations are used.

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## A POTENTIOMETRIC STUDY OF HEPATOFILAVIN

By F. J. STARE\*

(From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis)

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It has been shown that the flavins (alloxazine pigments) exhibit the properties characteristic of reversible oxidation-reduction systems. With lactoflavin, Kuhn and Wagner-Jauregg (1) discovered that, when the reduction is conducted in a strongly acid solution, an intermediate reddish color appears, which they interpreted as indicating the formation of a semiquinone. Barron and Hastings (2) in a preliminary report record the oxidation-reduction potentials of lactoflavin at different pH values. They observed that the slopes of the titration curves on the acid side lie between the slopes characteristic of 1 and 2 electron transfer systems, confirming the idea that the flavins belong among the two-step oxidation systems described by Michaelis (3). With a chloroform-soluble pigment of mammalian tissues which was probably identical with photoflavin, Bierich and Lang (4) obtained titration curves which gave no evidence of stepwise oxidation. Stern (5) has conducted a potentiometric study of the flavin derivatives, photoflavin and photo-yeast flavin. With these compounds he found that the slopes of the titration curves in the acid range increased with decrease in pH and that at very low pH values a break in the curve appears, indicating its separation into two 1-valent changes. He noted the appearance of a red color during the titration in this pH zone, which is good evidence for the existence of an intermediate of the semiquinone type. At neutral and alkaline zones the titration curves given by Stern resemble those of reversible organic systems having an electron transfer of 2.

The data here briefly recorded represent a study of the electro-

\* National Research Council Fellow.

chemical behavior of flavin prepared from horse liver, hepatoflavin. Although the number of the experiments is not large, they appear to outline this system sufficiently to permit a first comparison with the similar data of Stern for the photoflavins and with the preliminary data of Barron and Hastings for flavin from milk. The evidence now available points to a close similarity in the behavior of these three substances. A more detailed analysis of each will be necessary to disclose significant differences, if they exist.

The flavin used was prepared according to a procedure published elsewhere (6). Crystalline flavin was not used in these titrations, though it had been obtained from the solutions used for titration. The flavin solutions contained 1.17 mg. of nitrogen per cc. and a negligible amount of phosphorus. Examination of the solution with the spectrophotometer showed the absence of any substance absorbing light of wave-length greater than about 520  $\mu$ . From this wave-length absorption continued to the limit of the visible spectrum (470  $\mu$ ). This is typical of the visible part of the flavin absorption spectrum as reported by other workers. Such other substances as may have been present in the flavin solution were electromotively inactive and did not interfere with the titrations.

The oxidation-reduction potentials were measured by means of a Leeds and Northrup type K potentiometer. 5 cc. of the flavin solution were introduced into the titration vessel containing 50 cc. of the buffer solution. Two bright platinum electrodes were used. The titration vessel was placed in a constant temperature bath and connected by means of a 5 per cent agar, saturated KCl bridge to a saturated calomel half-cell. The solution was deoxygenated by bubbling through it nitrogen previously passed over hot copper gauze. The reducing solution for titrations was prepared as follows: 5 cc. of distilled water were added to 50 cc. of buffer solution and this solution was similarly deoxygenated. The reductant was then introduced, and deoxygenation continued. The solution was next transferred by nitrogen pressure to a microburette previously swept out with nitrogen. As reductants titanous sulfate (about 0.1 cc. of solution to 55 cc.) and sodium hydrosulfite (about 10.0 mg in 55 cc. of solution) were used. All titrations were carried out at 30°. At the end of each titra-

tion the pH of the reduced solution was determined with the hydrogen electrode. Owing to the fact that the flavin solution used in some of the titrations contained acetic acid, the pH values of the buffers were in some cases considerably modified by the addition of the flavin solution which contained acetic acid. In other cases the flavin solution was substantially neutral. In all cases the pH values determined after the titration was completed were assumed to be the level at which the oxidation-reduction had been carried out. The flavin solutions were of so low concentration (about  $10^{-5}$  M) in relation to buffer capacity that it was not considered necessary to make correction for the very small amount of acid formed by reduction. The observed potentials were converted to the normal hydrogen electrode standard, the basis of calculation being that the hydrogen electrode in 0.05 M potassium acid phthalate at 30° is -0.2386 volt (Clark).

The buffers used were as follows: Buffer 1, approximately N HCl, Buffer 2, approximately 0.1 N HCl; Buffer 3, 0.1 N  $\text{CH}_3\text{COONa}$  + 0.1 N  $\text{CH}_3\text{COOH}$ , Buffer 4, 500 cc of 0.2 M  $\text{KH}_2\text{PO}_4$  + 300 cc. of 0.2 M KOH + 200 cc. of 0.2 M KCl per liter; Buffer 5, 80 cc of M NaCl + 3 cc of 2 N NaOH + 50 cc of M  $\text{KHCO}_3$  per liter, Buffer 6, 6 cc of 1.99 N NaOH + 50 cc. of M  $\text{KHCO}_3$  per liter, Buffer 7, 30 cc of 1.99 N NaOH + 50 cc. of M  $\text{KH}_2\text{PO}_4$  per liter, Buffer 8, 45 cc. of 1.99 N NaOH + 50 cc. of M  $\text{KH}_2\text{PO}_4$  per liter, Buffer 9, M NaOH.

Table I gives the  $E'_0$  value at the various pH values, the  $\pi_A$  values, and the "index" potentials together with the  $E_2 - E_1$  values according to Michaelis' analysis (3).

The  $E'_0$  values are plotted against pH in Fig. 1. The lines are drawn with the theoretical slopes of 0.06 and of 0.03, and are so located as best to represent the several points. The  $E'_0$  values from 0.32 to 6.28 pH and from 10.7 to 13.38 pH give a good agreement to 0.06 slopes. The  $E'_0$  values at pH 8.95 and 9.3 lie on the 0.03 slope. The graphical analysis indicates a dissociation in the reductant at pH near 6.8 and in the oxidant at pH near 9.6. From similar analysis Stern's data for photohepato-flavin reveal a dissociation in the reduced form "near pH 7.7" and in the oxidized form "near pH 0.4 and 10."

At pH 4.13 and at values more acid than this a red intermediate between the greenish yellow oxidant and the colorless reductant

TABLE I  
Summary of Titration Data

Titration No	Buffer	Reductant	pH	$\pi_h$	$E'_0$	$E_{\text{index}}$	$E_2 - E_1$
					volt	volt	volt
1	HCl	$\text{Ti}_2(\text{SO}_4)_3$	0.32	-0.019	+0.165	0.033	+0.052
2	"	"	1.42	-0.085	+0.106	0.028	+0.033
3	Acetate	"	4.13	-0.248	-0.060	0.026	+0.025
4	Phosphate	$\text{Na}_2\text{S}_2\text{O}_4$	6.28	-0.377	-0.179	0.019	-0.027
5	Carbonate	"	8.95	-0.538	-0.275	0.026	+0.024
6	"	"	9.30	-0.559	-0.283	0.025	+0.020
7	Phosphate	"	10.70	-0.642	-0.352	0.023	+0.009
8	"	"	10.90	-0.655	-0.372	0.024	+0.014
9	NaOH	"	13.38	-0.804	-0.533	0.020	-0.016

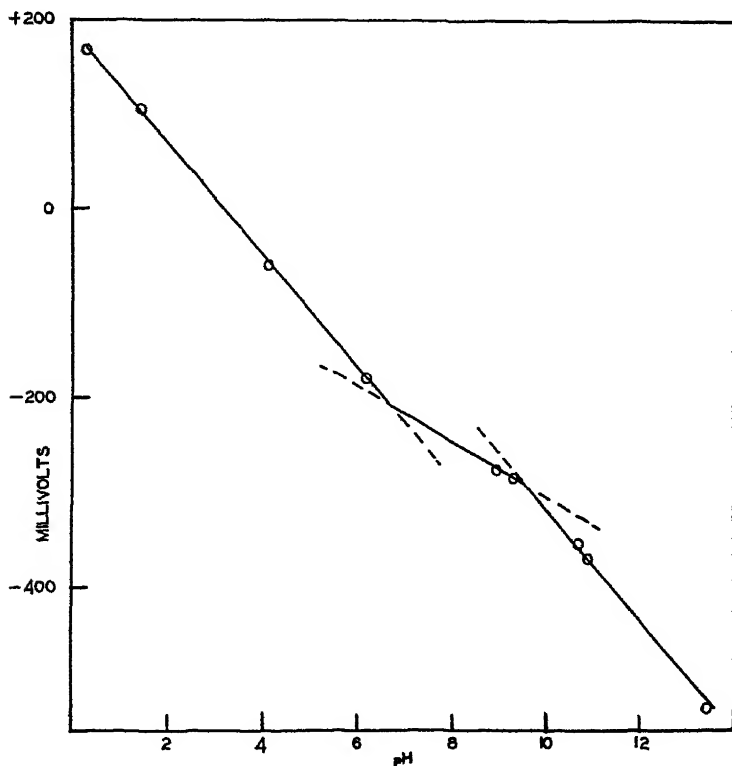


FIG. 1.  $E'_0$  plotted against pH value

was observed. The intensity of the intermediate red color increased with decrease in pH. This observation leads to the con-

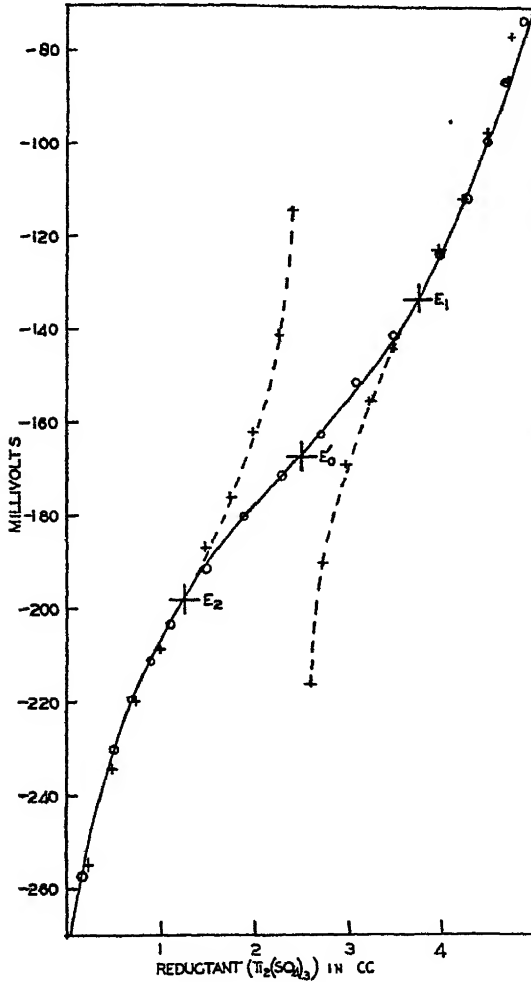


FIG 2. Titration curve of hepatoflavin at pH 0.32

clusion that two 1-valent oxidation-reduction steps probably occur in the acid regions. Since our titrations were not conducted



at pH values less than 0.32, the dissociation in the oxidant responsible for this effect cannot be precisely located from the data.

A composite graph giving all the titration curves was prepared, though it is not presented with this paper. A study of the "index" potentials obtained from the graph reveals that the system is composed of two 1-valent steps which in the strongly acid region begin to separate. At pH 0.32 the separation into two 1-valent steps is clearly evident and is accompanied by the distinct intermediate red color formation.

Fig. 2 gives the experimental and theoretical curves for the titration at pH 0.32. From the graph it is seen that each of the separate steps is symmetrical to the mid-point and that the theoretical curve for each step shows good agreement with the experimentally determined curve. At all higher pH values measured the curves overlap to such an extent that separation into two distinct 1-valent steps is obscured.

A comparison of the  $E_0$  value of these data with those of Barron and Hastings for lactoflavin and of Stern for photoflavin shows that all of the potentials fall in the same general region. Barron and Hastings give as the  $E_0$  value  $+0.187$  volt. The  $E_0$  value represented by the three titrations at pH 4.13 and below is  $+0.188$  volt.

The data now available do not permit further analysis of the hepatoflavin system, but reference should be made to another point. We include in Table I the "index" potentials (Michaelis) obtained graphically from the titration curves, and the corresponding values of  $E_2 - E_1$ , which represent the calculated degree of separation of the two  $E'_0$  curves for the two 1 electron steps. The sign and quantity of the  $E_2 - E_1$  values appear to suggest two regions within which the semiquinone may exist, one extending from about pH 4.4 to the acid side, and another from about pH 8.5 to high alkalinity, where another negative zone appears. Whether these suggestions are correct, further work must decide. If verified, the results would indicate several additional dissociations which are not disclosed by color change.

#### SUMMARY

The oxidation-reduction potentials of hepatoflavin have been measured at pH values from 0.32 to 13.38 by titration with reduc-

ing agents. Definite branching of the titration curves in the more acid regions into two 1-valent curves is indicated. Graphic analysis indicates ionization constants at pH values about 6.8 in the reductant and about 9.6 in the oxidant. An  $E_0$  value of +0.188 volt was obtained, which is rather close to the value reported for lactoflavin.

The author is indebted to Dr. P. A. Shaffer, Dr. P. W. Preisler, and Dr. E. S. Hill for their many helpful suggestions.

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## FURTHER STUDIES PERTAINING TO PROVITAMIN D OF PLANT AND ANIMAL SOURCES\*

BY R. M. BETHKE, P. R. RECORD, AND O. H. M. WILDER  
(From the Department of Animal Industry, Ohio Agricultural Experiment  
Station, Wooster)

(Received for publication, August 19, 1935)

The finding of Waddell (1) that the provitamins of cholesterol and ergosterol were not identical gave a new interpretation to the fundamental concept of vitamin D and offered a possible explanation of the many anomalous results concerning the efficacy of different vitamin D supplements. We were especially impressed with the report because it gave us a basis upon which to interpret our preliminary results on the comparative efficacy of vitamin D in metabolized (yeast) milk and irradiated milk for chicks. The observations of Waddell (1) were in such direct conflict with the fundamental concept on which much of the modern theory of vitamin D activity was based that it appeared sufficiently important to check his findings concerning the comparative efficiency of irradiated cholesterol, irradiated ergosterol, and cod liver oil, and to compare further the antirachitic efficacy of other irradiated products.

### EXPERIMENTAL

The basis of procedure throughout the several experiments was the correlated assays of the various antirachitic products on both rats and chicks. The rat assays were carried out according to the 10 day line test method applied to the split ends of the radius and ulna of animals fed the Steenbock and Black rachitic ration. The results were expressed in international rat units.

The chick assays were of the prophylactic type carried out with single comb white Leghorn chicks hatched from eggs laid by birds

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that received the same ration. The chicks were started on experiment when 1 day old and brooded in pens equipped with wire floors. In the experiments of Series I and II twenty chicks were started in each group and in Series III fifteen individuals were placed in each lot. Ash determinations were made on the individual alcohol- and ether-extracted moisture-free tibiae of ten to twelve representative birds from each group. Calcium determinations were made on the serum of the pooled blood according to the Clark-Collip method (2), and phosphorus determinations likewise, by the Bell-Doisy method (3).

The same basal ration as that described by Hart, Kline, and Keenan (4) was used in all the trials. It consisted of ground yellow corn 58, wheat middlings 25, domestic casein 12, calcium carbonate 1, calcium phosphate (tribasic) 1, dried yeast 1, iodized salt 1, and corn oil (Mazola) 1. In making additions to the basal ration in the first two experiments, we followed the practice of dissolving or suspending the preparations in corn oil so that 1 per cent of the oil introduced the desired amount of the vitamin D supplement. The same procedure was followed in the case of the reference cod liver oil and irradiated cottonseed oil in Series III. The other irradiated products used in the latter trial were incorporated by replacing an equivalent quantity of the basal ration.

The cod liver oil employed in Series I and II was a vitamin D concentrated oil and in Series III the U.S.P. reference oil. The irradiated ergosterol was obtained through the courtesy of Standard Brands, Inc., and the calciferol from The British Drug Houses, Ltd. The cholesterol was a pure product obtained from the Eastman Kodak Company and was irradiated for 1 hour in the dry state by spreading it out in a thin layer 15 inches below a 110 volt, Cooper Hewitt quartz mercury vapor lamp. The other irradiated products were similarly prepared. The butter fat and lard were irradiated in the molten state.

In Series I the irradiated cholesterol was fed at levels of 13.5, 27.0, and 54.0 rat units per 100 gm. of ration. There were also included two groups of chicks that received 13.5 and 27.0 rat units of vitamin D from cod liver oil and two groups that received 135 and 405 units as irradiated ergosterol in 100 gm. of ration. For comparative and control purposes, another group was fed an irradiated cholesterol concentrate supplied by Dr. Waddell. This

was added to the basal ration so as to furnish 27 rat units per 100 gm. of ration, which amount, according to Dr. Waddell, has proved to be near the minimum protective level in his laboratory.

The results presented in Table I show that equivalent rat units of vitamin D in irradiated cholesterol and cod liver oil were equally effective for the chick; whereas the vitamin D from irradiated ergosterol was decidedly less efficacious. The data also show that the vitamin D of the irradiated cholesterol concentrate and the irradiated cholesterol prepared in our laboratory were of compa-

TABLE I  
*Antirachitic Efficiency of Vitamin D from Cod Liver Oil, Irradiated Cholesterol, and Irradiated Ergosterol for the Chick*

Lot No	Vitamin D supplement	Rat units* per 100 gm ration	Average weight at 5 wks	Blood		Average ash in tibia
				Ca	P	
			gm	mg per 100 cc serum	mg per 100 cc serum	per cent
1	None	0 0	157 7	7 56	6 82	31 88 ± 0 49
2	Cod liver oil	13 5	314 8	10 80	7 50	41 84 ± 0 66
3	" " "	27 0	318.1	11 39	8 47	46 89 ± 0 25
4	Irradiated cholesterol	13 5	293 0	10 54	8 64	41 95 ± 0 58
5	" "	27 0	287 0	12 56	8 48	45 24 ± 0 39
6	" "	54 0	334 0	12 03	10 30	46 41 ± 0 33
7	" ergosterol	135 0	189 6	8 09	5 82	31 69 ± 0 32
8	" "	405 0	227 8	8 04	7 37	36 74 ± 0 78
9	" cholesterol concentrate	27 0	352 8	11 71	8 64	46 45 ± 0 19

\* International.

table efficacy. The blood serum analyses are in accord with the bone ash values and the usual findings of a significantly decreased calcium and lowered phosphorus content in the case of rickets in chicks. We have no explanation for the high phosphorus value in the case of Lot 6. Repeated determinations on the same sample of serum gave similar values.

It should be pointed out that the bone ash values for Lot 3 (cod liver oil) are, statistically, significantly greater than the average values for Lot 5, which received the equivalent rat unitage of vitamin D from irradiated cholesterol. The average growth of

the latter group (Lot 5) was also less than that of other comparable lots. Although we did not consider the above differences biologically significant because other groups (Lots 2 and 4) that received the same number of units of vitamin D, either in cod liver oil or irradiated cholesterol, did not show a similar difference, it was planned in part to repeat the experiment.

The plan of Series II was similar to that of Series I, except that the irradiated cholesterol and cod liver oil were fed at levels of 9.5 and 27.0 rat units per 100 gm. of ration and there were included two groups that received 135 and 405 units of vitamin D per 100 gm. of ration from calciferol and two more groups that received the same unitage from irradiated ergosterol. It was thought that some of the inactive resinous material in irradiated ergosterol might interfere with its antirachitic effectiveness and that calciferol representing a highly purified product free from these inactive materials might prove more efficacious.

The results of the experiment, which was of 5 weeks duration, are shown in Table II. It is very evident that the vitamin D of irradiated ergosterol and that of calciferol are of the same biological form which is antirachitically less efficient for chicks than the rat unit equivalent from cod liver oil or irradiated cholesterol. The data of both experiments confirm the observations of Waddell (1) that irradiated cholesterol had the same antirachitic efficiency as cod liver oil for chicks.

The fact that an irradiated sterol of animal origin and one of plant origin (ergosterol) were, antirachitically, not equally efficient for chicks, suggested a possible difference between plant and animal sources of the provitamin. This assumption had support in the review of Schonheimer (5) who pointed out that plant sterols in general are poorly, if at all, absorbed from the digestive tract. That ergosterol behaves similarly has been shown by Beumer and Hepner (6), who were unable to demonstrate any appreciable absorption of ergosterol by mice, and by the extensive experiments of Schonheimer and his associates (7) on dogs, rabbits, rats, and mice. On the contrary, Menschick and Page (8) and Schonheimer and Dam (9) reported that laying hens absorbed small amounts of ergosterol.

Steenbock and his associates (10) and Hess and Weinstock (11) as well as other investigators have shown that various plant and

animal products take on vitamin D activity, as determined with rats, when exposed to ultra-violet irradiation. Work with chicks has shown that certain irradiated plant and animal products are not effective in preventing rickets in this species. Payne and Hughes (12) observed that 2 per cent of irradiated cottonseed oil in the ration or the irradiation of the mash, which consisted of ground corn, wheat, oats, and meat scraps, was not satisfactory as a source of vitamin D for poultry. Likewise, Mussehl, Hill, and Rosenbaum (13) reported that ultra-violet irradiation of yellow or

TABLE II  
*Comparative Antirachitic Efficiency of Vitamin D from Cod Liver Oil, Irradiated Cholesterol, Irradiated Ergosterol, and Calciferol for the Chick*

Lot No	Vitamin D supplement	Rat units* per 100 gm ration	Average weight at 5 wks	Blood		Average ash in tibiae
				Ca	P	
				mg per 100 cc serum	mg per 100 cc serum	
			gm			per cent
1	None	0 0	148 6	7 45	5 44	31 91 ± 0 51
2	Cod liver oil concentrate	9 5	259 3	9 16	6 36	38 77 ± 0 74
3	" " " "	27 0	269 2	11 93	8 69	46 06 ± 0 27
4	Irradiated cholesterol	9 5	242 6	8 63	6 25	39 53 ± 0 73
5	" "	27 0	274 3	11 29	8 34	45 47 ± 0 30
6	" ergosterol	135 0	184 7	8 58	5 99	32 90 ± 0 55
7	" "	405 0	236 3	9 79	7 81	40 68 ± 0 69
8	Calciferol	135 0	170 9	7 88	6 70	32 06 ± 0 66
9	"	405 0	263 1	10 22	7 98	40 39 ± 0 84

\* International

white corn before grinding did not enhance its antirachitic values to any appreciable degree for chicks. The same investigators found that 3 per cent of irradiated corn oil or 20 per cent of irradiated dried liver in an otherwise complete ration did not protect chicks against rickets. Since no quantitative vitamin D assays were made on the irradiated products fed in the above chick experiments, it is impossible to postulate on the comparative antirachitic efficiency of the materials in question. However, in the light of present knowledge of vitamin D, the investigations suggest that the vitamin D in irradiated plant products might not



be as effective for chicks as animal sources of the vitamin. The unsuccessful attempt of Mussehl and his associates (13) to protect chicks against rickets with 20 per cent of irradiated liver meal appeared contrary to this assumption until we observed in our

TABLE III  
*Antirachitic Efficiency of Vitamin D in Irradiated Animal and Plant Products*

Lot No	Vitamin D supplement	Rat units* per 100 gm ration	Average weight at 28 days	Blood		Average ash in tibiae per cent
				Ca	P	
				mg per 100 cc serum	mg per 100 cc serum	
1	None	0 0	133 2	8 8	5 8	30 4 ± 0 32
2	Cod liver oil	6 7	175 2	9 4	6 2	35 6 ± 0 32
3	" " "	13 5	227 1	11 3	8 1	44 2 ± 0 58
4	Irradiated dried hog brains	4 0	154 6	8 6	6 0	32 8 ± 0 44
5	" " " "	8 0	201 6	10 2	6 7	36 7 ± 0 47
6	" " "	16 0	262 7	11 7	7 8	44 8 ± 0 58
7	" butter fat	5 5	183 7	9 6	5 4	35 3 ± 0 46
8	" " "	11 0	203 3	11 8	7 6	46 7 ± 0 29
9	" lard	8 0	184 8	10 4	5 9	39 7 ± 0 58
10	" "	16 0	225 2	11 5	7 0	44 9 ± 0 36
11	" cottonseed oil	13 5	148 1	7 5	5 1	30 6 ± 0 20
12	" " "	67 5	146 7	8 8	5 0	32 2 ± 0 57
13	" wheat middlings	13 5	127 2	7 7	5 2	30 3 ± 0 35
14	" " "	67 5	151 0	9 0	5 9	33 0 ± 0 66
15	" alfalfa leaf meal	13 5	139 5	7 9	4 5	29 3 ± 0 48
16	" " " "	67 5	156 5	8 5	5 4	32 1 ± 0 69
17	" dried mycelium†	67.5	155 2	8 6	5 8	33 5 ± 0 56
18	" " "	405 0	221 5	11 7	8 0	43 2 ± 0 94
19	" yeast	67 5	155 0	9 4	5 2	33 1 ± 0 96
20	" " "	405 0	234 0	11 5	6 8	44 4 ± 0 38

\* International

† *Aspergillus niger*.

laboratory that dried hog liver carefully irradiated for 1 hour contained less than 2 international rat units in 5 gm of liver. It also occurred to us that there might be a taxonomic relationship between the sterols of plants and their vitamin D effectiveness, since Gerard (14) had noted from his studies of the sterols in a

number of higher and lower plants that, in general, phytosterol is characteristic of the higher plants, or phanerogams, and ergosterol of the lower plants, or cryptogams, just as cholesterol is the typical sterol of animals.

To test out these hypotheses, different products of animal and plant origin were irradiated, carefully assayed for vitamin D on rats, and then fed to chicks in a rachitic ration. Dried hog brains, butter fat, and lard were the animal products chosen and cottonseed oil, alfalfa leaf meal, wheat middlings were selected as products representative of the higher plants, and dried yeast and dried fungus mycelium as those representing the lower form of plants. Two groups of chicks that received 6.7 and 13.5 rat units of vitamin D from reference cod liver oil per 100 gm. of ration were included for comparative purposes.

The results presented in Table III clearly show that the vitamin D of cod liver oil and the irradiated animal products (hog brains, butter fat, and lard) are antirachitically equally efficient and that the vitamin D in these products is definitely more efficacious for chicks than the rat unit equivalent form in the irradiated plant products. No difference was noted in the antirachitic effect of vitamin D in the products derived from the higher plants, as represented by irradiated cottonseed oil, irradiated wheat middlings, and irradiated alfalfa leaf meal, and the lower plant products, irradiated yeast and irradiated mycelium. The calcium and phosphorus contents of the blood serum were decreased in the case of inadequate intakes of vitamin D as determined by lowered bone ash values. The blood analyses, in general, were in agreement with the ash percentages observed in the tibiae.

The results offer definite evidence that the provitamin D of animal products and that of plant products are not identical and suggest that no taxonomic difference exists between the vitamin D efficiency of irradiated products from higher plants and irradiated plants of the lower botanical order.

#### SUMMARY

1. Data are presented which show that the vitamin D of irradiated cholesterol is more efficacious in preventing rachitic manifestations in chicks than the rat unit equivalent in irradiated ergosterol or in calciferol. It was observed that the vitamin D

of irradiated ergosterol and that of calciferol were of the same order of efficiency for chicks.

2 It is also shown that the antirachitic factor of irradiated cholesterol is as potent for chicks as the vitamin D of cod liver oil.

3. Since equivalent rat units of vitamin D from irradiated animal products (hog brains, butter fat, and lard) were more efficient antirachitically for chicks than vitamin D from irradiated plant products (cottonseed oil, wheat middlings, alfalfa leaf meal, yeast, and fungus mycelium), it is concluded that the provitamins of animal and plant products are not identical.

4. Evidence is presented suggesting that there is no taxonomic difference between the vitamin D efficiency of irradiated products from higher plants (cottonseed oil, wheat middlings, and alfalfa leaf meal) and irradiated plants (yeast and fungus mycelium) of the lower botanical order.

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## STUDIES OF THE ACID-BASE BALANCE OF THE BLOOD\*

### IV. CHARACTERIZATION AND INTERPRETATION OF DIS- PLACEMENT OF THE ACID-BASE BALANCE

BY NATHAN W. SHOCK AND A. BAIRD HASTINGS

(From the Lasker Foundation for Medical Research and the Department of  
Medicine of the University of Chicago, Chicago)

(Received for publication, August 26, 1935)

It is now well recognized that the conditions in the blood during the respiratory cycle, and in experimental or clinical acidosis or alkalosis, can be expressed in terms of three variables, *viz.* CO<sub>2</sub> tension, bicarbonate concentration, and pH, and that simultaneous determinations of any two of these variables fixes the third, in conformity with the Henderson-Hasselbalch equation. The relationships between these variables define what is now commonly known as the "acid-base balance" of the blood. Obviously, the determination of only one of the three variables is insufficient. It is now also well known that the acid-base balance may be displaced in different directions under various experimental and pathological conditions, and that the direction and extent of displacement are in themselves of physiological and clinical significance. The importance of these considerations was first pointed out in the classic paper of Van Slyke (23). Our knowledge of the subject has been made more exact by experiments upon blood *in vitro*, by experiments upon intact animals, and by observations upon the blood in pathological conditions. Since the subject has received thorough treatment by Peters and Van Slyke ((18) p. 868), an extensive review is unnecessary here.

With the methods previously available which have imposed limitations upon the number and frequency of observations, certain phases of the problem of the acid-base balance—more particularly, those having to do with the changes occurring with time

\* This work was supported by a financial grant from the Julius Rosenwald Fund.

following a disturbance of the normal relationships—have remained obscure. In an effort to make these phenomena accessible to study, the authors (21) have introduced a microtechnique, by which all of the data necessary for characterization of the acid-base balance may be obtained from analysis of minute samples of blood taken from the finger tips at intervals as short as 30 seconds. In addition, the authors (22) have devised a graphic method, depending upon the use of triaxial coordinate paper, for the representation and interpretation of shifts in the acid-base balance.

By the use of these methods the details of variations in the acid-base balance in the blood of normal individuals have been studied, which has resulted in a more complete understanding of the significance of shifts of the acid-base balance. In addition, studies of the rate of elimination of fixed alkali and fixed acid, by the normal individual, have been made. These comprise the contributions of the present paper.

#### *Theoretical*

Starting from normal conditions, acidosis may result from an excess of  $\text{CO}_2$  in the blood, alkalosis from a  $\text{CO}_2$  deficit. With no change in fixed acid or fixed base concentrations, the acid-base balance is a function of the  $\text{CO}_2$  tension and the bicarbonate concentration. When whole blood is titrated *in vitro* with  $\text{CO}_2$  the well known carbon dioxide absorption curve is obtained. From these considerations it would appear that, unless the organism responds to changes in  $\text{CO}_2$  tension with the entrance into or withdrawal from the blood stream of fixed acid or fixed base, any displacement of the acid-base balance of the blood *in vivo* as a result of  $\text{CO}_2$  excess or deficit should follow the path of the carbon dioxide absorption curve, and that any deviation from this path would indicate a change in fixed acid or base.

Acidosis may also result from excess of fixed acid or deficit in fixed base. Conversely, alkalosis may result from excess of fixed base or deficit of fixed acid. Under any of these conditions the  $\text{CO}_2$  tension or the bicarbonate concentration or both are altered by physiological processes in such a way that the acid-base balance is displaced, but may still be accurately characterized by the  $\text{CO}_2$  tension, bicarbonate concentration, and pH. The effects of adding fixed acid or fixed base to whole blood *in vitro* are known,

but much less is known as to the details of the response of the intact organism to the administration of these substances. The question of whether displacement of the acid-base balance as a result of the administration of fixed acid or fixed alkali occurs with or without change in  $\text{CO}_2$  tension is still an open one. It seems probable, however, from the results of previous work, that the typical physiological response to fixed acid excess is such that the  $\text{CO}_2$  tension and pH both decrease and, conversely, that the

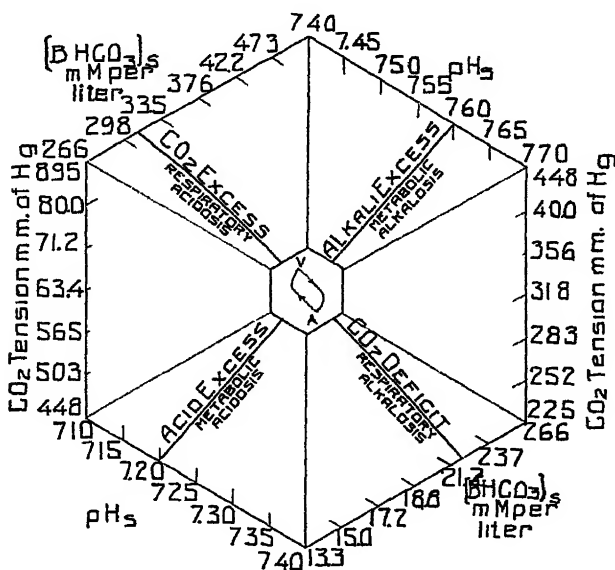


FIG 1 Acid-base chart showing four primary acid-base paths

response to fixed alkali excess is such that the  $\text{CO}_2$  tension and pH both increase

On the basis of these considerations a diagram has been constructed, representing, on triaxial coordinates, four hypothetical major pathways of displacement of the acid-base balance in the intact organism (Fig. 1). The line labeled " $\text{CO}_2$  excess" and " $\text{CO}_2$  deficit" represents an average  $\text{CO}_2$  absorption curve for oxygenated true plasma, as determined from *in vitro* experiments by Henderson and his coworkers (10). It is slightly curved to represent accurately the experimental data. The line labeled

"Acid excess" and "Alkali excess" has been drawn at right angles to the  $\text{CO}_2$  absorption curve. Drawn thus, it is consistent with known clinical and experimental observations that fixed acid excess results in decreased  $\text{CO}_2$  tension and pH as well as bicarbonate, and that fixed base excess results in increases of the three variables. The extent to which this hypothetical path is correct is one of the questions to be answered by the experiments of this paper.

As a further illustration of how a combination of changes of  $\text{CO}_2$  tension and of factors referable to the fixed acid of the blood may affect the acid-base path, the respiratory cycle of blood has been used as an example. The respiratory cycle of normal blood, plotted on the triaxial acid-base chart, takes the form indicated by the lines connecting the points *A* and *V* in Fig 1. The point *A* represents the acid-base balance of Bock's arterial blood, and the point *V* his mixed venous blood (10). It is apparent that the path from *A* to *V* not only indicates titration of the blood with  $\text{CO}_2$  but also the operation of a factor indicating an increase in fixed base. The latter is attributable to the reduction of the hemoglobin in the passage of the blood through the tissues, and the consequent lessening of its acidic strength. In passing from mixed venous to arterial blood, the same factors are effective but operate in the opposite direction. The small inner hexagon represents the normal area within which points denoting the acid-base balance of the blood of normal individuals may be expected to lie (22).

The present paper has a two-fold purpose: (1) the presentation of experimental data on the rate of change of various factors involved in the definition of the acid-base balance with particular attention to individual differences in the rate of return to normal after displacement; (2) the interpretation of the direction and extent of acid-base displacement and recovery in terms of the hypothetical pathways illustrated in Fig. 1.

#### *Methods*<sup>1</sup>

Blood samples were drawn and determinations of volume of cells, pH, and total  $\text{CO}_2$  were made (in triplicate) by the micro-

<sup>1</sup> Tables of the original data, of which only illustrative examples can be published here, may be obtained by applying to the authors

technique previously described (21). The  $\text{CO}_2$  tension of the blood,  $p\text{CO}_2$ , and the bicarbonate concentration of the plasma,  $(\text{BHCO}_3)_s$ , were calculated from the results of these determinations by use of a nomogram constructed for this purpose (8).<sup>2</sup>

The use of triaxial coordinates, above referred to, in studying the paths of displacement and of recovery, has been fully described in Paper III (22).

### *Rate of Change of Acid-Base Balance*

#### *Effects of Ammonium Chloride and of Sodium Bicarbonate*

Experiments upon the effects of fixed acid and fixed alkali on the acid-base balance were confined to the use of ammonium chloride and sodium bicarbonate, administered orally.

The effect of ammonium chloride ingestion on the acid-base balance of the blood of normal individuals has been studied by Gamble, Blackfan, and Hamilton (4), Haldane (7), and Koehler (13). Comparable experiments on the effect of sodium bicarbonate ingestion have been made by Davies, Haldane, and Kennaway (3), Koehler (13), Palmer, Salvesen, and Jackson (14), and Palmer and Van Slyke (15).

Previous investigators have adequately demonstrated that an acidosis with lowered  $\text{pH}_s$  and  $(\text{BHCO}_3)_s$  follows the ingestion of ammonium chloride, and an alkalosis with increased  $\text{pH}_s$  and  $(\text{BHCO}_3)_s$  follows the ingestion of sodium bicarbonate. Since their observations were made only at infrequent intervals after the ingestion of a single dose of the salt, their data are inadequate for the purpose of determining with accuracy the pathways followed, and the rates of change during displacement of the acid-base balance from, and recovery to, normal.

*Experimental Procedure*—Seven normal males served as subjects for thirty-eight experiments. The subject came to the laboratory, without breakfast, at 8.00 or 8.30 a.m. for each experiment, which was carried out as follows: (1) Samples of finger blood were drawn at 8.30 and 9.00 to serve as normal controls. (2) After drawing the blood sample at 9.00 a.m. the subject was usually given 5 or 10

<sup>2</sup> Although the method used in our work yields the  $\text{pH}$  and  $(\text{BHCO}_3)$  of plasma, it has been decided to use  $\text{pH}_s$  and  $(\text{BHCO}_3)_s$  throughout, in order to avoid multiplication of symbols. For the purposes of the paper, serum and plasma may be regarded as identical.



gm. of  $\text{NH}_4\text{Cl}$  in 300 to 500 cc. of  $\text{H}_2\text{O}$ , or 10 or 20 gm. of  $\text{NaHCO}_3$  in 300 cc of  $\text{H}_2\text{O}$ . (3) Blood samples were drawn at half hour intervals beginning at 9 30 a.m. and continuing until 12 30 p.m. (4) The subject ate lunch after the 12 30 blood sample was drawn (5) Blood samples were drawn at 1 hour intervals beginning at 1.30 and concluding at 6 30 p.m. A total of eighteen ammonium chloride experiments and twenty sodium bicarbonate experiments was completed.

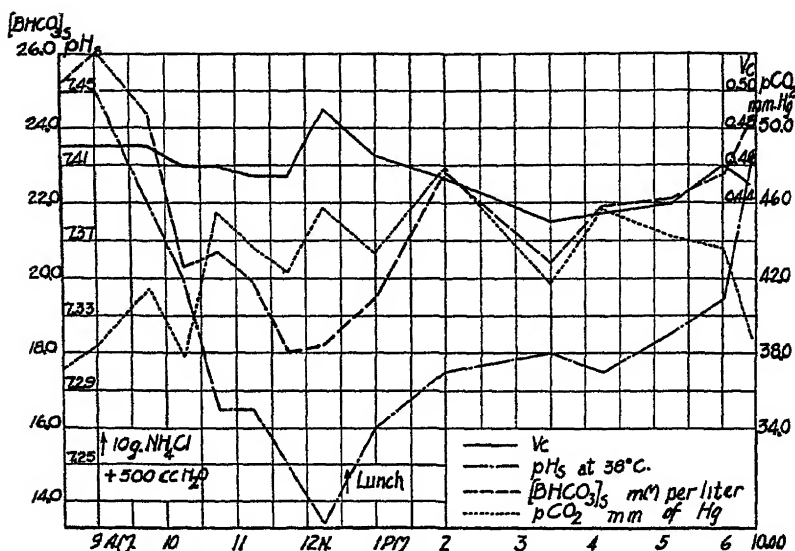


FIG. 2. Effect of  $\text{NH}_4\text{Cl}$  on the acid-base balance of the blood

In three additional experiments three 5 gm doses of ammonium chloride were given at 1 hour intervals in order to investigate the effect of successive doses. In six experiments a second dose of 20 gm. of  $\text{NaHCO}_3$  in 300 cc of  $\text{H}_2\text{O}$  was given at 1 30 p.m., and blood samples were drawn at half hour intervals during the afternoon as well as the forenoon. The final blood sample was drawn at 7.30 p.m. In cases where the results were not yet normal, another blood sample was obtained at 8 30 the following morning.

*Effects of Administration of Ammonium Chloride*—The data of each experiment— $V_c$ ,  $\text{pH}_s$ ,  $(\text{BHCO}_3)_s$ , and  $\text{pCO}_2$ —were first

plotted against time. While the general type of response following 10 gm. of ammonium chloride was the same in all individuals, as illustrated by a representative experiment (Fig. 2), certain variations were found in extent of displacement, time of maximum effect, and rate of recovery. Examination of the curves of the eighteen experiments with ammonium chloride indicated the following: (1) A progressive decrease in  $(\text{BHCO}_3)_s$  occurred, usually reaching a minimum between  $2\frac{1}{2}$  to 3 hours after the ingestion of the  $\text{NH}_4\text{Cl}$ . (2) The maximum decrease in pH, was 0.12 to 0.16 pH, for a dose of 10 gm. Dosages of 5 gm. produced their maximum effect in somewhat less time. (3) The rate of recovery was appreciably less than the rate of displacement, as shown by the rate of change of both pH, and  $(\text{BHCO}_3)_s$ . (4) There was often a rise in  $p\text{CO}_2$  during the period of displacement. When the point of maximum change in pH, and  $(\text{BHCO}_3)_s$ , was reached, however, the  $p\text{CO}_2$  usually decreased from 2 to 4 mm. of Hg. During the recovery period there was usually an additional decrease in  $p\text{CO}_2$  of from 3 to 5 mm. of Hg. These  $p\text{CO}_2$  changes will be brought out more clearly in the acid-base paths to be presented later.

The cumulative effect of small dosages of ammonium chloride was studied. When a single 15 gm. dose of ammonium chloride was given, the maximum displacement was not as great as when the same amount was given in three 5 gm. doses, the minimal  $(\text{BHCO}_3)_s$  being 18.7 and 14.8 mm per liter respectively. This observation is of importance in the use of ammonium chloride for therapeutic purposes.

*Effects of Administration of Sodium Bicarbonate*—The data were first plotted against time. Examination of the curves of the twenty experiments with sodium bicarbonate, one example of which is shown in Fig. 3, indicated the following: (1) The  $(\text{BHCO}_3)_s$  increased rapidly and reached a maximum within 1 to  $1\frac{1}{2}$  hours following sodium bicarbonate ingestion. It may be noted that the rate of change was more rapid and the maximum displacement was reached sooner than after ingestion of ammonium chloride. (2) The  $(\text{BHCO}_3)_s$  recovery curve was of a characteristic type, showing, in most instances, a rapid elimination from the blood for about  $1\frac{1}{2}$  hours following the peak response. During this period there was a decrease in  $(\text{BHCO}_3)_s$  of about 50 per cent from the

peak. The remainder of the bicarbonate was eliminated quite slowly, complete recovery sometimes not occurring until the following day. (3) The maximum increase in  $pH_s$  of about 0.10 to 0.16 pH for the given dosage of 20 gm. was reached between 1 and  $1\frac{1}{2}$  hours after ingestion. (4) In most individuals recovery of  $pH_s$  to normal values was complete within 2 to 3 hours. (5) In some of the experiments there was a slight increase in  $pCO_2$ , amounting to 4 to 10 mm of Hg, at the point of maximum increase in  $pH_s$  and  $(BHC\bar{O}_3)_s$ . (6) During the recovery period there was

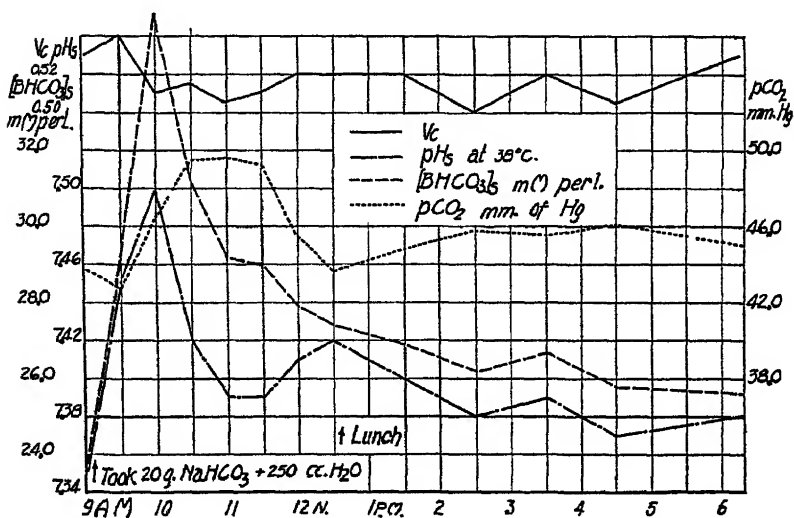


FIG. 3. Effect of  $NaHCO_3$  on the acid-base balance of the blood

sometimes a further increase in  $pCO_2$  of from 5 to 8 mm of Hg, although this was not an invariable result

Experiments were performed in which two doses of sodium bicarbonate were given on the same day. In addition to the features already pointed out, the following was observed: (1) The response to the second dose was usually greater than that to the first dose with respect to increase in both pH and bicarbonate. (2) The increase in  $pCO_2$  during the recovery period was particularly accentuated.

*Rate of Elimination of Excess Fixed Acid and Fixed Base*—Since our data permit the plotting of acid-base displacement and recov-

ery against time, it seemed desirable to find a means of characterizing, in quantitative terms, the rate at which the acid-base balance of the blood returns to its initial condition after displacement. It was felt that if such characterization were possible, individual differences in the ability of normal and pathological subjects to deal with abnormal amounts of fixed acids and alkalies might be revealed which were not shown by single acid-base determinations.

To plot simply the change of bicarbonate against time is insufficient, however, because, with increasing  $\text{pH}_s$ , more base is bound by the proteins of the blood, and the increase in bicarbonate observed is less than the actual addition of fixed base (or loss of fixed acid) which occurred. By utilizing the buffer value of whole blood, however, and correcting all observed values of  $(\text{BHCO}_3)_b$  to  $\text{pH } 7.4$ , the change in the amount of fixed acid or fixed base in the blood could be estimated. These calculations have been made, assuming the relation  $dB/d\text{pH} = 25$ , where  $dB/d\text{pH}$  represents the change in millimoles of base per liter of blood bound by blood buffers per unit change in  $\text{pH}$ , between the  $\text{pH}$  limits  $7.2$  and  $7.6$ . Since changes in percentage of cells were slight in each experiment, no attempt was made to correct for changes in the buffer value of the blood during the course of an individual experiment. The  $\text{BHCO}_3$  of the total circulating blood was then estimated by multiplying the  $(\text{BHCO}_3)_b$  at  $\text{pH}_s 7.4$  by the estimated blood volume, which was assumed to be  $0.077$  times the body weight of the subject. The apparent amount of additional fixed alkali or acid in the blood was estimated as the change in the total bicarbonate, compared with the initial value before the ingestion of the alkalinizing or acidifying salts. The percentage of the ingested acid or alkali present in the blood at definite times was then calculated by dividing the change in total bicarbonate by the millimoles of salt ingested, and multiplying by  $100$ . These percentages were plotted against time elapsed since administration of the salt. An example is given in Fig. 4.

These curves showed the approximate percentage of ingested fixed alkali or fixed acid present in the blood. They were characterized by two phases: an ascending portion in which the salt was being absorbed faster than it was being eliminated, and a descending portion in which the process of elimination from the

blood stream was predominant. The form of the curves during recovery suggested a relation to chemical reactions of the first order,  $dc/c = kdt$ , or  $\log c = kt + \text{constant}$ . By plotting the logarithm of the percentage change in bicarbonate against time, a linear relationship was obtained in which the slope,  $k$ , is tentatively called the *constant of elimination* (Fig 5). The significance of  $k$  is that it denotes the rate at which the logarithm of the percentage of circulating excess acid or alkali changes. Similar plots were made for all experiments, and the slope, or elimination constant  $k$ , was estimated for each.<sup>3</sup>

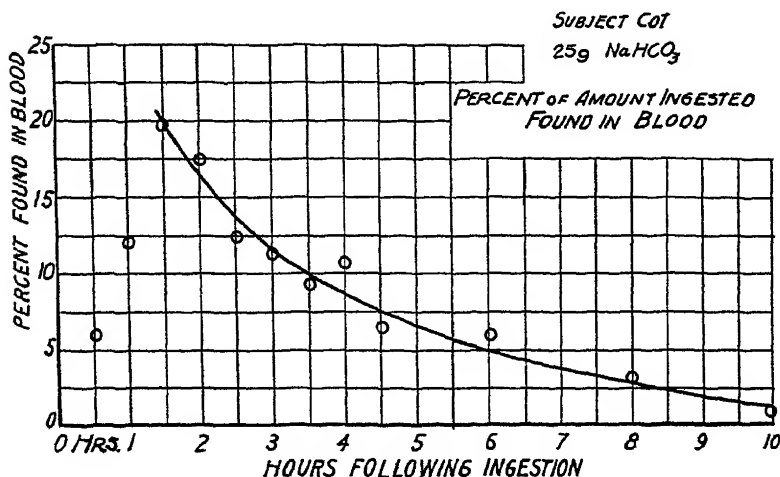


Fig. 4. Rate of disappearance of excess  $\text{NaHCO}_3$  from the blood

The individual values of  $k$  obtained in twenty-seven experiments on six individuals are listed in Table I. It may be seen (1) that reproducible elimination constants were obtained in duplicate experiments on the same individual when the same dosages of the same salt were used, (2) that varying the dosage did not

<sup>3</sup> It may be pointed out that plotting the logarithm of the change in  $(\text{BHCOS})_0$ , corrected to constant  $\text{pH}_0$ , against time will yield the same value for the elimination constant as the calculation just described. However, since our subjects were of different weights and were given different amounts of alkalinizing and acidifying salts, it was necessary, in studying the influence of these factors, to estimate the per cent of ingested salt which was circulating in each instance.

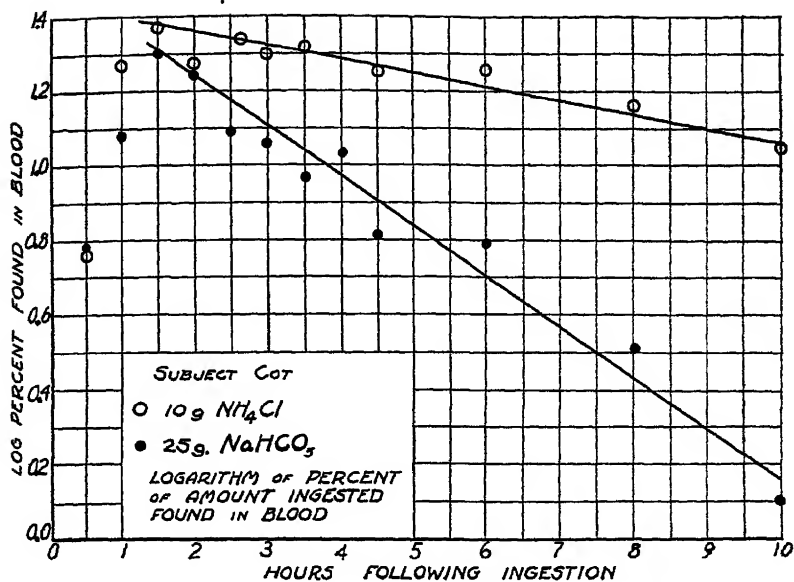


FIG 5 Examples of determination of elimination constants. For  $\text{NH}_4\text{Cl}$ ,  $k = -0.04$ ; for  $\text{NaHCO}_3$ ,  $k = -0.14$

TABLE I  
Elimination Constants

Subject	$\text{NH}_4\text{Cl}$					$\text{NaHCO}_3$				
	Dosage				Mean	Dosage			Mean	
	5 gm	10 gm	15 gm	20 gm		10 gm	20 gm	25 gm		
JB	-0.10	-0.08	-0.08		-0.08	-0.08	-0.07		-0.07	
JE		-0.08			-0.06		-0.05		-0.06	
		-0.06					-0.07	-0.05		
		-0.06								
LM	-0.11	-0.08			-0.08	-0.11	-0.11		-0.11	
AM	-0.03	-0.04		-0.04	-0.04	-0.11	-0.12		-0.11	
	-0.06				-0.06		-0.05		-0.04	
	-0.07						-0.03			
CT		-0.04			-0.04			-0.14	-0.14	

materially influence the value of  $k$ , and (3) that in two of the six subjects studied, the elimination constant following ingestion of  $\text{NaHCO}_3$  was strikingly greater than that following  $\text{NH}_4\text{Cl}$

Although the series is too small to permit statistical treatment, it would appear that the individual subjects varied with respect to the mean value of  $k$  obtained. These differences in elimination constants were more marked with  $\text{NaHCO}_3$ , than with  $\text{NH}_4\text{Cl}$ . It is probable that such differences with respect to the excretion of acid and alkali in pathological conditions may be of clinical significance.

The smaller the dose of ammonium chloride, the greater was the per cent of circulating excess acid at the time of maximum acid-base displacement. This is shown by the experiments on JB and AM in Table II. Whether or not this is also true for sodium bicarbonate awaits further experimentation. It was also usually

TABLE II  
*Percentage of Acid or Alkali Ingested Found in Blood at Time of Maximum Acid-Base Displacement*

Subject	Dose		NH <sub>4</sub> Cl Maximum displacement		Subject	Dose		NaHCO <sub>3</sub> Maximum displacement	
	gm	mM	hrs	per cent		gm	mM	hrs	per cent
JB	5	94	0 5	29 5	JB	20	238	1 0	17 2
"	10	187	2 0	20 9	"	20	238	1 0	19 3
"	15	281	3 0	13 9	LM	10	119	1 0	32 6
AM	5	94	2 0	39 0	"	20	238	1 5	22 0
"	10	187	2 0	14 5	SS	20	238	1 5	19 0
"	15	374	2 5	11 8	AM	20	238	1 0	13 6

observed that the point of maximum acid-base displacement occurred after a longer time had elapsed following the ingestion of ammonium chloride than following an equivalent dose of sodium bicarbonate.

#### *Effects of Rebreathing and Overbreathing*

*CO<sub>2</sub> Excess*—The changes in the acid-base balance of the blood produced by increasing the CO<sub>2</sub> tension of the inspired air have been described by several investigators (3, 11, 20). However, the question of whether the changes are simply those to be expected if blood is titrated with carbon dioxide, or whether changes in fixed acid or base are involved, remains an open one (18). The purpose of the experiments to be described in this section was to

titrate the blood *in vivo* with  $\text{CO}_2$  and study both the nature of the changes produced in the acid-base balance of the blood and the temporal relationships involved in these changes.

*Experimental Procedure*—A series of twenty-four experiments was carried out, with six normal males as subjects. The subject rebreathed through a closed system of about 35 liters of oxygen for 15 to 18 minutes, during which time the per cent of  $\text{CO}_2$  gradually rose to approximately 9 per cent. Samples of blood were drawn at 2 minute intervals and analyzed as previously described. Samples of the air in the tank were analyzed at 2 minute intervals for the per cent of  $\text{CO}_2$  by the Haldane apparatus for gas analysis.

*Physiological Effects*—In all subjects the respiration increased gradually in rate and amplitude, as has been reported in numerous experiments (3, 16, 19). Toward the end of the rebreathing period, the respiration rate and amplitude reached uncomfortable proportions, finally making continuation of the experiment impossible.

*Blood Changes*—The data from all experiments were plotted against time, as illustrated by a representative experiment in Fig. 6. The  $\text{pH}_s$  showed a decided decrease during rebreathing. In most experiments the drop began after 2 minutes of rebreathing, although in the first experiments with some subjects the  $\text{pH}_s$  slightly increased during the first 2 minutes of rebreathing. This increase in  $\text{pH}_s$  may have been produced by overbreathing on the part of the subject. The  $\text{pH}_s$  continued to fall throughout the rebreathing period at a rate of 0.02 to 0.04  $\text{pH}_s$  per minute, reaching values of 7.18 to 7.20 in all experiments. When rebreathing was stopped, the  $\text{pH}_s$  rapidly returned to normal. In fact, in some experiments the  $\text{pH}_s$  was higher 2 minutes after the cessation of rebreathing than 4 minutes later.

The  $\text{pCO}_2$  of the blood increased during rebreathing at a positively accelerated rate, rising from normal values of 40 to 45 mm of Hg to a maximum of 70 to 77 mm. In some experiments there was a slight decrease in  $\text{pCO}_2$  during the first 2 minutes of rebreathing.

Not much change occurred in  $(\text{BHCO}_3)_s$  for the first 4 to 8 minutes of rebreathing. Subsequently the  $(\text{BHCO}_3)_s$  increased at the rate of 0.5 to 1.0 mm per liter per minute until the end of the rebreathing period.



*CO<sub>2</sub> Deficit*—It has been well established that with overventilation the following changes take place. As the  $p\text{CO}_2$  of the alveolar air—and, consequently, of the blood—decreases, the pH, increases and the  $(\text{BHCO}_3)_s$  decreases. Whether or not a decrease in the level of the  $\text{CO}_2$  absorption curve also occurs appears to depend

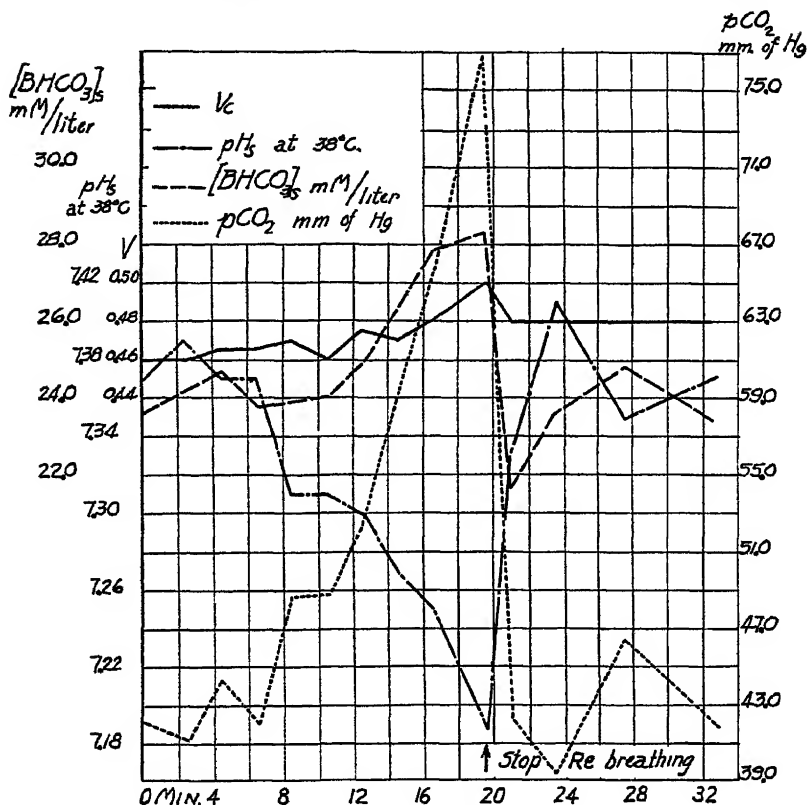


FIG. 6. Effect of rebreathing on the acid-base balance of the blood

to a large extent upon the length of time the hyperpnea is maintained (5, 12, 17). The evidence on these points has been thoroughly reviewed by Peters and Van Slyke ((18) p 954), and will not be repeated here. Since doubt exists as to the direction, rate, and extent of the changes produced both during the period of displacement and the period of recovery, the acid-base microtechnique

was employed to study these questions (21). In addition, acid-base paths were determined in experiments where overbreathing was combined with fixed alkali and fixed acid excess.

*Experimental Procedure*—A series of twenty-four experiments was carried out on six normal males. The subjects came to the laboratory at 8.30 a.m. without breakfast. First, a normal control sample of blood was drawn; then the overbreathing was begun with the subject lying on his back. The breathing was carried on as forcibly as possible, with emphasis on expiration. The overbreathing period varied in extent from 6 to 20 minutes. Blood samples were drawn from the finger-tips at 2 minute intervals during the overbreathing period, and for the first 10 minutes of the recovery period. Subsequent samples were drawn at intervals of 3 to 8 minutes for the succeeding 20 minutes.

*Physiological Effects*—The physiological effects of hyperventilation have been repeatedly described (1, 2, 6, 16). The extent of the changes induced in these experiments may be judged from the fact that in all subjects, except one, definite signs of tetany, including carpopedal spasm, were observed.

*Blood Changes*—Results from all experiments were plotted as in Fig. 7. The changes in  $pH$ ,  $pCO_2$ , and  $(BHC_3)_s$  occurring during overbreathing and during recovery may be summarized briefly.

The  $pH$ , increased rapidly—in many experiments as much as 0.04 to 0.06  $pH$ , per minute—during the first 3 or 4 minutes of overbreathing. In experiments where the overbreathing was continued for a longer time, this initial period was followed by one in which the rate of increase was not so rapid. Perhaps the most striking observation of this series of experiments was the rapidity with which the  $pH$ , returned to normal following the cessation of overbreathing. While individuals differed in their speed of recovery, the  $pH$ , dropped 0.10 to 0.24  $pH$ , units during the first 2 minutes after cessation of hyperventilation, depending on the extent of displacement. In most experiments the  $pH$ , had returned practically to normal within the first 4 minutes after overbreathing was stopped. The remaining decrease in  $pH$ , took place at a diminishing rate, complete recovery being attained in 15 to 20 minutes.

The inverse relationship between the  $pCO_2$  and  $pH$ , was strikingly illustrated. Overbreathing produced a rapid fall in  $pCO_2$ ,

amounting to 4 to 6 mm. of Hg per minute in most experiments. In all the experiments the rate of decrease in  $p\text{CO}_2$  showed a negative acceleration—the first 2 minutes of overbreathing producing the greatest change per minute. Values of  $p\text{CO}_2$  as low as 15 to 20 mm. of Hg were reached in most of the experiments. After

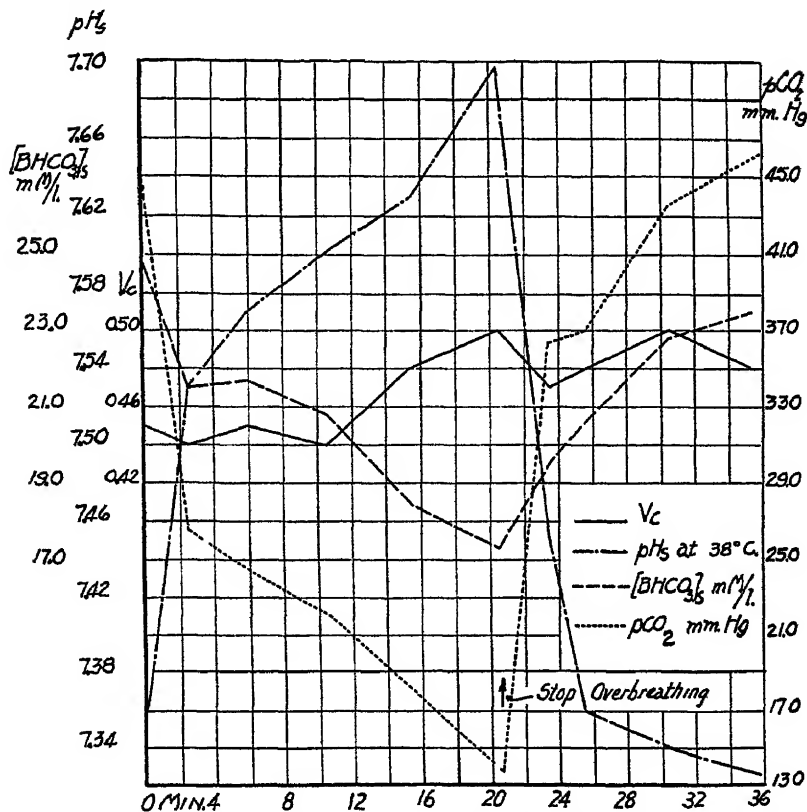


Fig 7 Effect of overbreathing on the acid-base balance of the blood

overbreathing, the  $p\text{CO}_2$  of the blood rose as much as 25 mm. of Hg in 2 minutes. The subsequent rise was at a decreasing rate, complete recovery requiring 15 to 25 minutes.

In all experiments, the  $(\text{BHCO}_3)_s$  decreased slowly. In most cases this decrease progressed at a practically constant rate of from 0.2 to 0.7 mm per liter per minute. The actual decrease amounted

to from 4 to 7 mm, depending upon the length of time the overbreathing was continued. The bicarbonate content of the serum increased as soon as the  $p\text{CO}_2$  started to rise following the cessation of overbreathing, and returned to a value about 1 to 2 mm per liter below the initial value within 15 to 20 minutes.

#### *Acid-Base Paths of Displacement and Recovery*

*Administration of Ammonium Chloride*—The data obtained from the time curves were plotted on triaxial coordinates, in order to investigate the paths of acid-base displacement. Each point on such a chart represents the acid-base condition of the blood at a given time. As stated before, the time interval between the first ten points is  $\frac{1}{2}$  hour, while that between subsequent points is 1 hour.

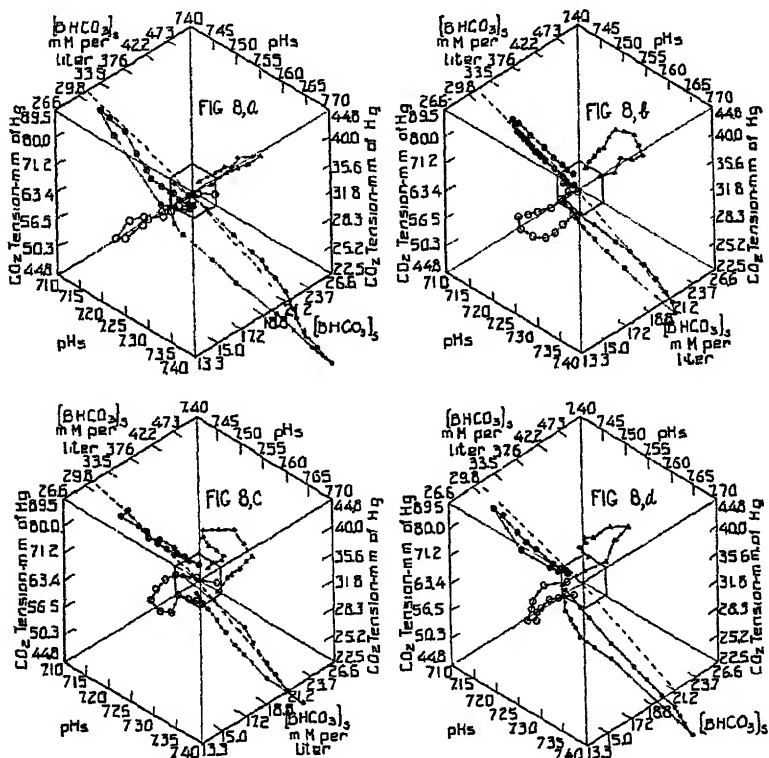
Fig. 8 illustrates the types of paths encountered in four subjects. The large open circles represent experimental observations following the administration of  $\text{NH}_4\text{Cl}$ . These diagrams show that after the ingestion of ammonium chloride the change in the blood during the period of displacement was in the direction of decreased pH, and  $(\text{HCO}_3)_s$ , without much change in  $p\text{CO}_2$ . In some instances, however, there was an immediate increase in  $p\text{CO}_2$  during the first half hour following the ammonium chloride ingestion.

After the point of maximum displacement was reached, there was a period of decreasing  $p\text{CO}_2$  which resulted in an increase in pH. The subsequent return to normal varied somewhat in the direction taken, but in general may be said to have followed a course of increasing pH,  $(\text{HCO}_3)_s$ , and  $p\text{CO}_2$ . These events may be described as indicating that there was a period of displacement without "compensation" of pH, followed by a period of recovery with compensation (23).

*Administration of Sodium Bicarbonate*—In Fig. 8 the triangular points represent data on four individuals following the administration of  $\text{NaHCO}_3$ . From an examination of the acid-base paths of all the experiments with sodium bicarbonate the following statements may be made. (1) The path of displacement of the acid-base equilibrium by the ingestion of sodium bicarbonate was linear when plotted as shown here. This line of displacement tended to remain practically parallel to the constant  $p\text{CO}_2$  lines. (2) In all twenty experiments there was an increase in  $p\text{CO}_2$  after the

maximum  $\text{pH}_s$  and  $(\text{BHCO}_3)_s$  were attained. There was then a period of increased  $p\text{CO}_2$  which caused a swing in the path toward the initial  $\text{pH}_s$  value. The subsequent return to the normal condition took place with decreasing  $(\text{BHCO}_3)_s$ ,  $p\text{CO}_2$ , and  $\text{pH}_s$ .

Thus it may be said that during the period of displacement of



FIGS 8, a to 8, d Four primary acid-base paths of displacement and recovery determined on four normal human subjects. For explanation of symbols, see the text.

the acid-base balance with fixed base excess, there was apparently no compensation of  $\text{pH}_s$ , but that during the recovery period compensation occurred (23).

**$\text{CO}_2$  Excess**—In Fig. 8 the acid-base paths during rebreathing and recovery are indicated as circled crosses. In these graphs each

point represents a blood determination at the constant interval of 2 minutes. The dotted line in the charts is the  $\text{CO}_2$  titration curve of normal human blood, plotted from the data of the *in vitro* experiments of Henderson and his collaborators. The graphs show that the slopes of the displacement paths of normal individuals were quite constant, and closely paralleled the  $\text{CO}_2$  titration curve of normal human blood. The slopes of the recovery curves were not sufficiently different from those of the displacement curves to indicate that there had been any significant change in fixed acid or fixed base as a result of  $\text{CO}_2$  excess.

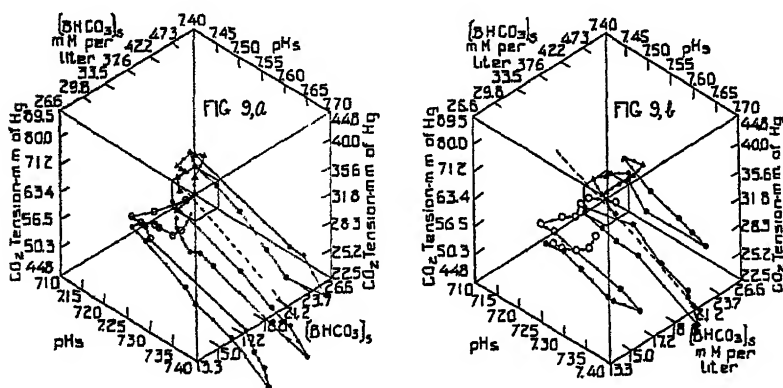
*CO<sub>2</sub> Deficit*—In Fig. 8 the solid black points represent data obtained at 2 minute intervals during overbreathing experiments. The linearity of the displacement path and the constancy of its slope, not only with respect to different experiments on the same subject but also for different subjects, were striking. These experiments showed that while the displacement path proceeded along the  $\text{CO}_2$  titration curve during the early stages, after continued overbreathing there was an appreciable increase in fixed acid of the blood which persisted for a much longer period than the  $\text{CO}_2$  effects. The recovery paths followed a line parallel to but lower than the paths of displacement

Experiments were also performed in which the period of overbreathing was preceded by a single dose of either sodium bicarbonate (25 gm) or ammonium chloride (10 gm), producing changes in the fixed acid-base condition of the individual as previously reported, in order to determine the effect of such displacement on the path produced by overbreathing. The character of the changes resulting from overventilation was unaffected by fixed acid and fixed base displacement, although their positions on the acid-base chart were of course altered. In Fig. 9 the solid triangles indicate the results of blood analyses made at half hour intervals following the ingestion of 25 gm of  $\text{NaHCO}_3$  in 500 cc. of  $\text{H}_2\text{O}$ ; the open circles, the results following the ingestion of  $\text{NH}_4\text{Cl}$ . The solid circles are determinations made at 2 minute intervals during overbreathing and recovery. These results show strikingly the differentiation between metabolic and respiratory factors involved in the regulation of the acid-base balance of the blood.

Furthermore, from Fig. 9 it can be seen that the recovery path

swings toward the direction of increased fixed acid in all the experiments. This may be taken as evidence for the presence of increased amounts of acid in the blood, amounting to about 1 to 2 mm per liter, probably formed as a result of the increased muscular work and the reduced oxygenation of the tissues following the diminished blood flow resulting from the overbreathing

In view of the fact that tetany occurred in all overbreathing experiments on all subjects save one (AHM), regardless of whether they were normal or in a condition of fixed acid or alkali excess, it is believed that the excessive decrease in  $p\text{CO}_2$  is to be viewed as the contributing cause, rather than the increase in  $\text{pH}_s$ . Subjects



FIGS. 9, a AND 9, b Complex acid-base paths Respiratory alkalosis superimposed upon metabolic acidosis and alkalosis For explanation of symbols, see the text

with alkalosis produced by the ingestion of sodium bicarbonate often had a  $\text{pH}_s$  higher than 7.50 without exhibiting tetany; whereas subjects with acidosis produced by the ingestion of ammonium chloride and then subjected to overbreathing developed tetany with  $\text{pH}_s$  of 7.50 to 7.55. On the other hand, when the  $p\text{CO}_2$  of the blood was diminished to approximately 20 mm, tetany ensued irrespective of the  $\text{pH}_s$ .

It may also be pointed out that in these experiments instances were encountered in which the  $\text{pH}_s$  was normal, but the  $(\text{BHC0}_3)_s$  and  $p\text{CO}_2$  were markedly decreased. (See Fig. 9.  $\text{pH} = 7.40$ ,  $(\text{BHC0}_3)_s = 18$  mM per liter,  $p\text{CO}_2 = 30$  mm. of Hg) Such an

observation might be regarded as a condition of compensated acidosis. As a matter of fact, in this instance it represents a combination of two abnormal conditions, which may be designated as *metabolic acidosis* and *respiratory alkalosis*.

*Major Acid-Base Paths*—The outstanding points of similarity in the paths illustrated in Fig. 8 are: (1) The paths following  $\text{CO}_2$  excess and deficit correspond closely with the  $\text{CO}_2$  absorption curve of blood, represented by the broken line on the charts. (2) The paths of recovery following  $\text{CO}_2$  excess are, within the limits of observation, identical with the paths of displacement. (3) The paths of recovery following  $\text{CO}_2$  deficit are slightly displaced to the left, indicating the acquisition of some fixed acid by the blood. (4) The paths of displacement and recovery following fixed acid and base excess are approximately at right angles to the  $\text{CO}_2$  absorption curves. (5) The fixed acid excess recovery curve is always displaced to the right of the displacement curve to a greater or less extent, indicating a respiratory adjustment leading to a lower  $p\text{CO}_2$  and higher  $\text{pH}_s$ . (6) The fixed base excess recovery curve is always displaced to the left, indicating a respiratory adjustment leading to a higher  $p\text{CO}_2$  and lower  $\text{pH}_s$ .

The points of dissimilarity are that the direction of displacement produced by fixed acid and base excess varies in different experiments, although it is usually approximately along a constant  $p\text{CO}_2$  line. The paths of recovery also vary in different individuals, from one approximating a constant  $p\text{CO}_2$  line to one making a right angle with the path produced by changes in  $\text{CO}_2$  tension.

*Interpretation of Abnormal Acid-Base Conditions*—Four major paths of displacement and recovery of the acid-base balance have been found experimentally, corresponding approximately to the hypothetical paths illustrated at the beginning of this paper (Fig. 1). It has been seen that the effects of the four major influences upon the acid-base balance, when acid-base data are plotted on triaxial coordinates, are, in typical instances, sufficiently separated from one another to permit the characterization of deviations from the normal condition. Pathological conditions affecting the acid-base balance may, on the basis of the considerations just cited, be classified under the following four main divisions.

*Metabolic Acidosis*—By this is meant a condition of fixed acid



excess or fixed alkali deficit without respiratory complications. It is indicated in Fig. 1 by the line labeled "Acid excess." This type of acidosis may be the result of excessive production of fixed acid, as in diabetes, or inadequate excretion of fixed acid, as in nephritis. It may be pointed out that anoxia leading to lactic acid accumulation causes an acid-base displacement in the direction of metabolic acidosis.

*Metabolic Alkalosis*—By this is meant a condition of fixed alkali excess or fixed acid deficit without respiratory complications. It is indicated in Fig. 1 by the line labeled "Alkali excess." This type of alkalosis may be the result of loss of fixed acid, as in pyloric obstruction, or excessive absorption of fixed base, as in the treatment of gastrointestinal disorders with basic salts.

*Respiratory Acidosis*—By this is meant a condition of  $\text{CO}_2$  retention resulting from inadequate removal of  $\text{CO}_2$  from the lungs. It is indicated in Fig. 1 by the line labeled " $\text{CO}_2$  excess." This type of acidosis may be the result of obstruction in the respiratory system, inadequate movement of the respiratory muscles, inadequate lung surface available for aeration, or a decreased coefficient of diffusion through the pulmonary epithelium. In the presence of inadequate oxygenation of the blood in the lungs and any factor leading to metabolic acidosis, the displacement follows closely the  $\text{CO}_2$  absorption curve of the blood *in vitro*, and is equivalent to titrating the blood with  $\text{CO}_2$ .

*Respiratory Alkalosis*—By this is meant a condition of excessive loss of  $\text{CO}_2$  resulting from overventilation. It is indicated in Fig. 1 by the line labeled " $\text{CO}_2$  deficit." This type of alkalosis may be the result of excessive activity of the respiratory mechanism originating in the central nervous system. It is also observed in hyperthermia and in exercise accompanied by an increase in temperature. The direction of acid-base displacement follows closely the  $\text{CO}_2$  absorption curve of blood *in vitro*. Recovery to normal may be accompanied by slight displacement toward the direction of metabolic acidosis, owing probably to the entrance into the blood stream of lactic acid.

*Combinations of Two Factors*—Sometimes two or more factors tending to displace the acid-base condition from normal may operate simultaneously. For example, if one finds a decreased  $(\text{HCO}_3)_s$  and  $p\text{CO}_2$ , but normal  $\text{pH}_s$ , a condition of metabolic

acidosis and respiratory alkalosis is indicated. On the other hand, the acid-base displacement may be in a direction of increased  $p\text{CO}_2$ , decreased  $\text{pH}_s$ , and decreased  $(\text{BHCO}_3)_s$ . This would indicate a condition of metabolic acidosis and respiratory acidosis. Both of these conditions have been observed in dogs by Hastings and Steinhaus (9), the first, when the animals swam in water at a temperature of  $40^\circ$  and the second when the water was at a temperature of  $15^\circ$ .

It is hoped that the accumulation of further observations on the acid-base balance and the simultaneous study of clinical symptoms associated with the activity of the respiratory and cardiovascular systems will lead to a better understanding of the etiology of acid-base disturbances.

#### SUMMARY

The acid-base balance of the blood of normal individuals has been studied at frequent intervals under a variety of experimentally produced abnormal acid-base conditions. From the results it has been concluded: (1) that individuals may be characterized in terms of the rate at which the acid-base balance of the blood is restored to normal after experimental displacement; (2) that abnormal acid-base conditions may be characterized in terms of the physiological factors involved.

The significance of these conclusions for the interpretation of pathological acid-base conditions has been pointed out.

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## STUDIES IN AMINO ACID METABOLISM

### I. FATE OF GLYCINE, *DL*-ALANINE, AND *D*-ALANINE IN THE NORMAL ANIMAL

BY JOSEPH S. BUTTS, MAX S. DUNN, AND LOIS F. HALLMAN

*(From the Department of Biochemistry, University of Southern California School of Medicine, and the Chemical Laboratory, University of California at Los Angeles, Los Angeles)*

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If one is willing to accept the hypothesis that any substance which is convertible to "extra sugar" in a phlorhizinized animal is a source of glycogen and ketolytic material, and that under such an abnormal régime the pathway of metabolism of the substance is the same as is found in the normal animal, then there can be little doubt but that alanine and glycine are excellent glycogen formers.

Ringer and Lusk (1) have shown a complete conversion of *DL*-alanine to urinary glucose under phlorhizin poisoning. Dakin (2) obtained the same result after *L*-alanine, using a similar technique. Ringer and Lusk (1) were also able to show the same complete conversion when glycine was fed. These experiments carried out under such an unphysiological procedure do show a possibility of conversion but shed no light on the behavior of these compounds in the normal animal.

A few workers have attempted to study glycogen formation after the administration of amino acids to normal animals. Of the various papers, as Wilson and Lewis (3) have pointed out, in only two previous to the publication of their paper, do the experiments appear to have been satisfactorily controlled. Simon (4), who studied glycogen formation after giving leucine to animals which had been rendered glycogen-free by fasting and by the use of strychnine, concluded that this amino acid was not a glycogen former. The second paper in which the work seems to have been well controlled is Pfluger and Junkersdorf's (5) in which glycine was given to dogs which had received phlorhizin to deplete the

glycogen deposits. In one case a definite increase in liver glycogen was noted, while in a second experiment no rise in this constituent was found. In two experiments on normal fasted animals definite glycogen formation was evident.

Wilson and Lewis (3), using rats as experimental animals, have studied the liver and body glycogen after oral administration of glycine, *d*- and *dl*-alanine, *d*-glutamic acid, and *l*-leucine. Of these substances they report that only *d*-alanine, *dl*-alanine, and *d*-glutamic acid showed glycogen deposits significantly higher than the control levels, while glycine and *l*-leucine were negative in this respect.

Another method which may be used to study sugar formation from amino acids is to try their effect on the condition of ketonuria. Because so few of the lower animals develop such a condition, it is necessary to resort to an artificial ketosis. Such a method has been used by Butts and Deuel (6), in which sodium acetoacetate is fed in sufficient amount to cause considerable quantities of the acetone bodies to be eliminated in the urine. This condition responds to feeding of carbohydrate or precursors of glucose (Shapiro (7)) in much the same way as a ketonuria induced by fasting or carbohydrate-free diet in man.

Since there seems to be considerable doubt in regard to the quantitative relationships, it seemed to us to be a field worthy of investigation. The synthesis of the pure amino acids and their resolution into active isomers are being carried out by one of us (M. S. D.), while the metabolism of these compounds is being studied by another author (J. S. B.). This paper is the first of a series in which we hope by comparison of glycogen formation and evaluation of the acids as to their ketolytic or ketogenic properties to place the ones available in their correct category in metabolism.

#### EXPERIMENTAL

The study of the metabolism of these amino acids is divided into two parts. The first part is in relation to their ability to form glycogen; the second, to test their effect on the condition of ketonuria.

Male rats ranging from 200 to 250 gm. in weight were used for the glycogen studies. After a preliminary 48 hour fast, the rats were fed the sodium salts of the amino acids by stomach tube.

In the case of glycine 2.00 mg. per sq.cm. of surface area was the dose employed. The alanines were fed on the basis of carbon content, which would mean 1 mole of alanine for each 1.5 moles of glycine. This amounted to 1.58 mg per sq cm of body surface. The surface area was calculated according to the formula of Lee (8). 8 hours after the feeding of the amino acids, the livers were removed and glycogen determined according to the technique described elsewhere (Deuel *et al* (9)).

The ketolytic properties of the various amino acids have been studied after developing an artificial ketonuria, by the technique of Shapiro (7), which consists of feeding 1.5 mg. of sodium acetoacetate (calculated as acetone) per sq cm. and superimposing upon this the substance whose ketolytic activity is to be studied. The total amount of the acids given daily in two divided doses in the ketolytic experiments was the same, except in the experiments reported in Table IV, as was employed in the glycogen studies. In the ketolytic experiments reported in Table IV one-half this amount was the quantity given. Female rats were used for this part of the work. Urine collections were made every 24 hours as described elsewhere (6), and analyses were carried out for total acetone bodies by the Van Slyke method and total nitrogen according to the Kjeldahl technique. It seemed important to us, in view of the different rates of absorption of the various amino acids (10), to determine whether or not absorption was complete after 8 hours. Accordingly, the gastrointestinal tract was removed, in some cases from the anus to the esophagus, in others from the cecum to the esophagus. Before cutting, the tract was clipped off proximally to the section. A large syringe was fitted onto the lower end of the tract and 50 cc. of hot water forced through the gut and stomach. The washings were made acid with acetic acid and heated to boiling; after this 10 cc. of 20 per cent trichloroacetic acid were added to insure complete precipitation of the protein, after which the solution was made up to 100 cc and filtered. Aliquots of this solution were used for the determination of amino nitrogen according to the Van Slyke technique.

### Results

It will be noted that for economy of space as a rule only summary tables are reported. However, the ratio of the mean differ-

ence to the probable error of the mean difference is calculated. In the glycogen determinations it is computed for various groups, one against the other. When the difference of this ratio exceeds 3, the differences between the averages of the comparable groups are considered statistically significant (9).

In the control group of twenty males the values ranged from 0.00 to 0.56 with an average of 0.24 per cent. This figure is in close agreement with the results of Wilson and Lewis (3) who report 0.21 per cent liver glycogen, although their animals had only a 24 hour fast. Also they fail to report the sex of their animals.

TABLE I

*Liver Glycogen Determinations on Male Rats Receiving, after a 48 Hour Fast, Either Glycine (2 Mg.), dl-Alanine (1.58 Mg.), or d-Alanine (1.58 Mg.) per Sq.Cm (8 Hours between Feeding and Killing)*

Substance fed	No of rats	Liver glycogen, per cent			P.E. of mean	Mean difference/ P.E. of M.D. compared with			Per cent above mean compared with		
		Minimum	Maximum	Mean		(I)	(II)	(III)	(I)	(II)	(III)
Control (I)	20	0 00	0 71	0 24	0 023					95	100
Glycine (II)	10	0 24	1 14	0 60	0 057	5 92			100		100
dl-Alanine (III)	10	0 66	2 44	1 14	0 122	7 37	4 06		100	90	
d-Alanine (IV)	10	1 25	3 71	2 15	0 117	16 02	11 89	6 01	100	100	100

In Table I there are given the results on liver glycogen formation after feeding the sodium salts of glycine, *d*-alanine, and *dl*-alanine.

In Table II are reported the results on residual amino nitrogen found in the washings of the gastrointestinal tract. The only experiment which would indicate any retention of the amino acid is one result on an animal which received glycine. Even in this case there was approximately 87 per cent absorption, the other two experiments showing 97 and 96 per cent. The alanines gave somewhat better absorption values, in both experiments more than 98 per cent.

A control experiment was carried out to determine the validity of the method. *dl*-Alanine was given by stomach tube and the gastrointestinal tract immediately removed and the washings carried through the usual procedure. When increasing amounts

were given, the per cent recovery was less, as, when 25.16 mg. of nitrogen were fed, 102.8 per cent was recovered. Twice this amount fed resulted in 94 per cent recovery. After 2.5 times the first amount, a recovery of 91.5 per cent was found.

When the amino acid was carried through the precipitation but without feeding, a 100 per cent recovery was found, showing that none of the amino nitrogen was lost by the technique employed in protein precipitation.

After glycine, the average value for liver glycogen amounted to 0.60 per cent (range 0.24 to 1.14); following *dl*-alanine 1.14 per

TABLE II

*Amino Nitrogen Content of Washings of Gastrointestinal Tract of Male Rats Fasted 48 Hours with and without Alanine or Glycine*

Substance	No. of rats	Absorption time	Amino N fed	Amino N recovered			
				Minimum	Maximum	Mean	
		hrs	mg	mg	mg	mg	per cent
Control*	5	0	0	2 44	3 53	2 90	
<i>dl</i> -Alanine*	1	0	25 16			26 10	102 8
"	2	0	50 32	46 80	47 90	47 35	94 0
"	1	0	62 90			57 40	91 5
Glycine	3	8	122 00	3 44	16.10	7 78	6 14
<i>dl</i> -Alanine	3	8	80 00	0 73	1 38	1 02	1 27
<i>d</i> -Alanine	3	8	80 00	1 10	1.54	1 25	1 56
Control	4	8	0	1 11	1 33	1.13	

\* Control experiments to determine the accuracy of the method.

cent (0.32 to 2.44); and after *d*-alanine 2.15 per cent (1.25 to 3.71). The statistical treatment of the data shows that the mean differences in each case are significantly different from the control value, and that the same is true for the average of *d*-alanine when compared with glycine or *dl*-alanine.

Generally speaking the ketolytic experiments followed along in the same order one finds in evaluation of the various substances as glycogen formers; namely, glycine the poorest from the standpoint of both a glycogenic and ketolytic compound, with *dl*-alanine somewhat better in both respects, and with *d*-alanine decidedly the best of all. These results are given in Tables III to V.



## DISCUSSION

In view of the results of Wilson and Lewis (3) the fact that glycine gives any glycogen is surprising. However, the value of 0.60 per cent in comparison with a control value of 0.24 per cent is quite definite. More weight may be given to these data when statistically compared with the control. When the mean difference is divided by the probable error of the mean difference, a value of 5.92 is found. Further, with only one animal receiving glycine was the liver glycogen as low as the mean glycogen content of the control group, and this was identical, 0.24 per cent. Only one control was higher than the average value of the animals receiving glycine.

Although Wilson and Lewis (10) report that this acid is not as readily absorbed as some of the others, notably alanine, yet the amount we fed would fall well within the range one would expect to be absorbed in 8 hours. The determinations carried out to ascertain residual amino nitrogen gave essentially blank values, although in one case the value after glycine seemed unusually high. On this basis we concluded complete absorption.

In the ketolytic experiments there can be no doubt but that the excretion of acetone bodies is decreased when glycine is fed along with the sodium acetoacetate. The average daily excretion of total acetone bodies when glycine was fed expressed as gm. per sq.m. (calculated as acetone) for the 5 days is as follows: 3.31, 3.06, 3.16, 3.41, 3.63, against control values for litter mates of 6.29, 8.71, 10.00, 10.72, 10.44. When compared statistically every day, the values were found to be significantly lower than the control.

In the experiments with *DL*-alanine the liver glycogen was found to be considerably higher than after glycine containing an equivalent amount of carbon. The value of 1.14 per cent for ten animals again showed statistical significance against both the control and animals receiving glycine, with none of the individual experiments as low as the average of the control group and only one lower than the average of the animals given glycine. The ketolytic experiments showed essentially the same results; namely, that in comparison to glycine *DL*-alanine is a much better ketolytic agent. For the 5 days, values of 2.55, 2.29, 2.00, 1.65, and 1.34 gm. of total acetone bodies per sq.m. were found which are considerably

TABLE III  
*Average Acetone Body Excretion (in Gm. per Sq. M. of Body Surface) in Urine of Fasting Female Rats Receiving 1.5 Mg. of Sodium Acetoacetate and Either 2 Mg. of Glycine or 1.68 Mg. of Alanine Per Sq. Cm. Daily*

Experimental day	1		2		3		4		5	
	Mean	M.D./P.E. (M.D.)	Mean	M.D./P.E. (M.D.)	Mean	M.D./P.E. (M.D.)	Mean	M.D./P.E. (M.D.)	Mean	M.D./P.E. (M.D.)
Control	6.29(7)		8.71(6)		10.00(7)		10.72(7)		10.44(6)	
Glycine	3.31(8)	5.80	3.06(8)	18.51	3.16(8)	19.70	3.41(7)	16.68	3.63(5)	11.42
d-Alanine	2.55(8)	7.28	2.29(8)	20.80	2.00(8)	22.98	1.65(8)	20.70	1.34(8)	16.49
d-Alanine	2.95(3)		1.57(3)		1.44(3)		1.32(3)		1.45(2)	

Figures in parentheses represent the number of animals included in the average.

TABLE IV

*Acetone Body Excretion of Female Rats (in Gm. per Sq M. of Body Surface)  
Receiving 1.5 Mg. of Sodium Acetoacetate per Sq Cm per Day and  
Either d-Alanine or dl-Alanine (0.80 Mg. per Sq Cm per Day)*

	Experiment No	Surface area	Acetone body excretion		
			1st day	2nd day	3rd day
d-Alanine	176	292	2 23	1 64	1 06
	177	279	1 60	1 30	1 25
	178	266	1 89	2 82	1 50
	179	271	1 00	1 34	0 83
	180	264	0 96	1 54	1 58
	181	265	1 72	2 04	1 50
	182	264	1 00	2 00	1 97
	183	264	0 97	1 21	0 69
	184	260	1 33	1 80	0 84
Average . . . . .		268	1 41	1 74	1 25
dl-Alanine	185	277	2.19	2 78	2 84
	186	288	3 83	4 47	5 77
	187	273	1 16	1 38	1 12
	188	268	1 12	1 25	1 39
	189	294	1 26	2 37	1.93
	190	267	1 94	1 20	1 24
	191	252	2.12	2.64	2 54
	192	271	1 58	2 16	1 21
	193	266	2 68	2 58	2 23
Average . . . . .		273	1 99	2 31	2 25
Control	194	277	4 30	5 92	8 22
	195	266	2 09	4 22	5 12
	196	268	1 44	2 25	3 83
	197	262	4 27	7 79	9 42
	198	264	3 58	6 15	8 34
	199	271	4 72	10 22	10 39
	200	252	2 72	4 00	6 83
	201	271	1 30	2 80	8 22
	202	285	2 68	3 55	4 89
Average.		268	3 01	5 21	7 25

lower than the values noted above for the animals receiving glycine and much lower than those for the controls. A comparable number of animals was used in each group. Statistical evaluation of these data is given in Table III.

The most surprising results were found after the administration of *d*-alanine. In comparison to a liver glycogen value of 1.14 per cent after *dl*-alanine, 2.15 per cent of glycogen was present after feeding *d*-alanine, with *none* of the individual values as low as the averages of the animals receiving *dl*-alanine.

One would expect that, if the glycogen figures are correct, *d*-alanine should show a greater ketolytic ability than *dl*-alanine. In Table III are recorded the results of three experiments for 4 days and for two animals on the 5th day. The number of tests is too small to mean very much, but in 3 out of the 5 days the mean acetone body output after the isomer is lower than the mean after the racemic mixture.

TABLE V

*Average Acetone Body Excretion of Female Rats (in Gm. per Sq.M. of Body Surface) Receiving 1.5 Mg. of Sodium Acetoacetate per Sq Cm per Day and Either d-Alanine or dl-Alanine*

Twenty-seven experiments; nine animals for each day.

Substance fed	Acetone bodies			P E of mean	M D / P E (M D) compared with	
	Minimum	Maximum	Mean		(I)	(II)
Control (I) . . . . .	1 44	10 39	5 13	0 353		
<i>dl</i> -Alanine (II)	1 12	5 77	2 18	0 127	7 88	
<i>d</i> -Alanine (III) . . . .	0 83	2 83	1 47	0 063	10.23	5 00

Since the results on *d*-alanine were too few to allow definite conclusions, we felt it necessary to carry out an experiment with a large group of animals. Such a study is reported in Table IV. The averages for total acetone bodies, expressed in gm. per sq.m. per day for the 3 days, are for *d*-alanine 1.41, 1.74, and 1.25 as against 1.99, 2.31, and 2.25 for *dl*-alanine, and 3.01, 5.21, and 7.25 for the control animals. When compared statistically, taking the total of nine animals for 3 days or a total of twenty-seven experiments, one finds, although the ketosis tends to become more severe with each day, causing a great difference between maximum and minimum values, that the results are significant. The evaluation is reported in Table V.

It will be noted that although the quantity of the alanines is only one-half the amount fed in the experiments reported in Table

III, the acetone body levels are lower. The animals used were much younger and this probably explains the low results, both after the alanines and with the control animals. However, since litter mates were used throughout in each series of tests and all experiments were run simultaneously, we believe these results to be valid.

Although experiments have not been carried out on *l*-alanine, it seems reasonable to assume that the superior glycogenic and ketolytic behavior of *d*- over *dl*-alanine is probably to be ascribed to the fact that the dextro component alone is active. *dl*-Alanine therefore should be only half as effective as an equal amount of the *d* isomer in glycogenic and ketolytic action.

In addition to the experimental data given in this paper to substantiate this statement, an analogy to the variability in the behavior of the lactic acid offers further support. Mandel and Lusk (11) reported a significant difference in the metabolism of *d*- (sarco-) and *dl*-lactic acid. They believe that under phlorhizin poisoning *d*-lactic acid may be completely converted into glucose. However, when *dl*-lactic acid was fed, only a partial conversion to glucose occurred. They interpret their results as follows:

"That under favorable conditions half or more of the lactic acid ingested could be converted to dextrose, while the rest burned and reduced proteid metabolism, suggested the idea that it might be possible that the *l*- and *d*- components behaved differently in the diabetic organism, the latter being converted into dextrose and the former burning in part, and in part being converted into dextrose."

Although these experiments were carried out under phlorhizin, Cori (12) has shown that such a difference holds in the normal animal and that only the *d*- (sarco-) lactic acid is effective in glycogen formation, while its isomer *l*-lactic acid is devoid of this property. These results have been confirmed by Shapiro (7), who further showed that a similar variability held in their ketolytic activity. The first compound was effective in causing acetone bodies to be burned, while the latter one was not.

One of the possible pathways for the metabolism of alanine assumes a conversion to lactic acid. We believe this conversion of *d*-alanine to *d*-lactic acid and *l*-alanine to *l*-lactic acid is the explanation for the superior glycogenic property of *d*-alanine over that of the racemic mixture. If this be true, it would rule out the

possibility of pyruvic acid or any other substance, which would cause a loss of asymmetry of the central carbon atom, being in the pathway of metabolism. Otherwise, one alanine or one lactic acid would be just as effective as its isomer.<sup>1</sup>

In comparison to the results one expects from glycine fed to an animal under phlorhizin poisoning, in which this compound is reported to go 100 per cent to "extra sugar" (1), these figures on glycogen formation are rather difficult to explain. We believe these values indicate that one must use caution in interpreting the results from phlorhizin experiments and that the use of phlorhizin gives a qualitative indication of what happens normally but is entirely lacking in a quantitative aspect. Further, as Wilson and Lewis (3) have pointed out, there are several factors which may tend to reduce glycogen formation: first, the slower rate of absorption, although this seems an unlikely factor, since absorption was practically complete after 8 hours; second, the increased specific dynamic action of glycine over alanine.

It is rather difficult to explain the discrepancy between our results and those of Wilson and Lewis (3). The only valid explanation is that these investigators failed to separate their animals as to sex. This might account for the differences in glycogen levels as reported in the two papers.

#### SUMMARY

1 Glycine has been shown to be definitely glycogenic. Further proof of its conversion at least to a ketolytic material is afforded by showing its effect on a ketonuria.

2 *dl*-Alanine is very much superior to glycine, both as a glycogen former and a ketolytic agent.

3 *d*-Alanine is approximately twice as effective as a glycogen former as *dl*-alanine and definitely superior to the latter compound as a ketolytic agent.

4 It is suggested that as far as glycogenic properties are con-

<sup>1</sup>Abderhalden and Tetzner (13) have reported that when *dl*-alanine was administered either subcutaneously or orally only the *d* form was utilized, with the *l* form failing to undergo metabolism. As Dr. C. G. King has suggested, this lack of utilization of the unnaturally occurring isomer is possibly a question of enzymatic specificity and probably will hold with all of the unphysiological isomers of the amino acids.

cerned only the *d*-alanine is effective, while in the racemic mixture the *l*-alanine seems to be very ineffective in this regard.

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## FEEDING EXPERIMENTS WITH MIXTURES OF HIGHLY PURIFIED AMINO ACIDS

### VII. THE DUAL NATURE OF THE "UNKNOWN GROWTH ESSENTIAL"\*

By MADELYN WOMACK† AND WILLIAM C. ROSE

(From the Laboratory of Physiological Chemistry, University of Illinois,  
Urbana)

(Received for publication, August 26, 1935)

It has been demonstrated repeatedly in the preceding papers of this series that young rats fail to maintain themselves upon diets carrying a mixture of nineteen amino acids in place of proteins. On the other hand, the addition to such a ration of a concentrate of the monoamino acids is followed immediately by growth. These facts were interpreted as proving that "growth-promoting proteins contain at least one essential dietary component other than the twenty known amino acids" (Rose, 1931).

Hitherto we have referred to this substance as a single entity. No conclusive proof was obtained for its multiple nature, despite numerous attempts to purify it by fractional crystallization of the protein concentrates, fractional precipitation by various reagents commonly employed in protein analyses, and fractional distillation of the esters of the amino acids (*cf.* Caldwell and Rose (1934)).

On the other hand, our suspicions have been aroused frequently during the past 2 years by the fact that procedures which theo-

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The data in this paper were presented in abstract before the American Society of Biological Chemists at Detroit, April 11, 1935 (Rose, W. C., McCoy, R. H., Meyer, C. E., Carter, H. E., Womack, M., and Mertz, E. T, *J Biol Chem*, 109, lxxvii (1935)).

† The experimental data in this paper are taken from a thesis submitted by Madelyn Womack in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Physiological Chemistry in the Graduate School of the University of Illinois



retically should have effected a concentration of the unknown were accompanied instead by a *loss* of activity. These observations appeared to necessitate one of two conclusions, either that the growth stimulant consisted of more than a single compound, and that a partial separation had been accomplished by the procedures employed; or that the substance, if a single entity, became less stable when freed of some of its contaminants. If the latter possibility were correct, autoxidation would seem to be the simplest explanation. Hence, a concentrate was prepared by a procedure in which each step, as far as practicable, was carried out in an atmosphere of nitrogen (Caldwell and Rose, unpublished data). However, the crude material thus obtained showed no greater growth-stimulating effect than similar fractions prepared in contact with air.

Such was the baffling situation when the problem was reattacked in the fall of 1934. In most of our earlier work the first step in preparing the active material involved the exhaustive extraction of a hydrolyzed protein with aqueous butyl alcohol. In order to remove most of the growth stimulant from a 12 kilo lot of hydrolyzed protein, eighteen to twenty-two extractions with 30 to 40 liter portions of butyl alcohol are required. Experience has shown that the amino acids recovered from the first few extracts manifest much less growth-stimulating action, per unit of weight, than do those from the later extracts. This is due to the diluting effect of the large quantities of very soluble amino acids which pass into the first few portions of butyl alcohol. Therefore, the material from these early extracts is usually discarded. The amino acids recovered from the later extracts are combined, and subjected to methods designed to concentrate the unknown to a greater extent.

Inasmuch as such large volumes of butyl alcohol are necessary for the complete removal of the growth stimulant, the possibility occurred to us that one or two reextractions of an aqueous solution of the amino acids recovered from the *later* butyl alcohol extracts might accomplish a concentration of the unknown, if it were a single entity, or effect a separation of the two components, if the active material possessed a dual nature. The success of this simple procedure exceeded our expectations. Not only did it accomplish a division of the crude material into two fractions, neither of which, without the other, sufficed to render the diet

satisfactory; but it led promptly to the identification of both growth-limiting factors. The methods employed and the identity of the component showing the greater solubility in butyl alcohol are discussed below.

#### EXPERIMENTAL

In most of our investigations casein has been employed in the preparation of the active fractions. 2 years ago the supplementing action of about twenty proteins was investigated by incorporating them in the diet, one at a time, in 5 per cent concentration, in place of an equivalent quantity of the amino acid mixture (Halmbacher and Rose, and Behrens and Rose, unpublished data). Of the proteins tested, blood fibrin proved to be the most effective. At that time, we were under the impression that our basal diet was deficient in only one substance. The existence of two types of deficiency obviously invalidates these tests, and necessitates their repetition. They are mentioned here because of the fact that fibrin was used in this and other of our later investigations.

The preparation of the two active fractions was carried out as follows: 12 kilos of crude, dry fibrin<sup>1</sup> were ground in a mill, and extracted with ether. After removal of the ether, the protein was hydrolyzed with sulfuric acid in the usual manner (Berg and Rose, 1929), and the acid was quantitatively removed by the addition of barium hydroxide. The filtrate and washings from the precipitate of barium sulfate were combined and concentrated *in vacuo* to a volume of about 14 liters. On standing, 1560 gm. of the less soluble amino acids precipitated and were discarded. The solution was then extracted twenty-two times with 30 liter portions of butyl alcohol. The extractions were carried out at room temperature in large crocks in which the mixtures were violently agitated by two motor-driven stirrers operating in opposite directions. The amino acids from Extracts 1 to 5 (537 gm.) were discarded, inasmuch as they showed little growth-stimulating effect. The material recovered from Extracts 6 to 22 (666 gm.) was dissolved in 4 liters of water, and reextracted twice with 5 and 6 liter portions, respectively, of butyl alcohol. For this purpose the mixtures were vigorously agitated on a shaking machine. The first reextraction

<sup>1</sup> We are indebted to Dr. David Klein of The Wilson Laboratories for supplying a large amount of blood fibrin for these studies.

was carried out for half an hour, and yielded 20.8 gm. of butyl alcohol-soluble material. The second reextraction was continued for 1 hour. From the butyl alcohol layer 53.6 gm. of amino acids were obtained.

In order to concentrate further the material in the water solution, the latter was evaporated *in vacuo* to a small volume. The amino acids which precipitated were removed and recrystallized from water. A fraction of 105 gm. was thus obtained and discarded. The combined filtrates were then treated with an excess of basic copper carbonate, and the mixture was heated for a short time on a steam cone. On cooling, the insoluble copper salts separated and, together with the excess copper carbonate, were removed by filtration. To the filtrate 25 gm. of basic copper carbonate were added, and the whole was evaporated to dryness *in vacuo*. The residue was treated with 1 liter of hot water, thoroughly shaken, concentrated *in vacuo*, and allowed to stand overnight in an ice box. On filtering, 85.5 gm. of insoluble copper salts were removed. All of the water-insoluble copper salts were discarded, inasmuch as Caldwell and Rose (1934) have shown that they carry no growth-stimulating material.

Finally, the water-soluble copper salts were treated with hydrogen sulfide and filtered. The solution of free amino acids was concentrated *in vacuo*, transferred to an evaporating dish, and dried in a vacuum oven. Thus, three fractions of amino acids were recovered from the 666 gm. of material present in the original butyl alcohol Extracts 6 to 22 inclusive; namely, 20.8 gm. from the first reextraction with butyl alcohol, 53.6 gm. from the second reextraction, and 300 gm. from the water-soluble copper salts of the aqueous layer.

Preliminary growth experiments showed that none of the three preparations, when added singly to the basal diet, exerted more than a very slight growth-stimulating effect. Therefore, the three possible combinations carrying two fractions each were tested. The results demonstrated conclusively that a separation of the butyl alcohol-soluble material into two active fractions had been accomplished. The material obtained from the first butyl alcohol reextraction contained most of one factor with minute traces of the second, while the material recovered from the water-soluble copper salts carried most of the other factor contaminated

with small amounts of the first. Evidently, one substance is quite soluble in butyl alcohol under the conditions described. This was temporarily designated as Unknown I. The second is removed very slowly from its aqueous solution by extraction with butyl alcohol. This was temporarily designated as Unknown II.

In view of the results of the preliminary trials, more extended feeding tests were conducted. The composition of the diet employed is shown in Table I. The "supplement" consisted of 4

TABLE I  
*Composition of Diet\**

	Diet C-7
	gm.
Amino acid Mixture I† . . . . .	22 4
Supplement . . . . .	4 0
Methionine ( <i>dl</i> -) . . . . .	0 6
Glucosamine hydrochloride ( <i>d</i> -) . . . . .	1 0
Sodium bicarbonate . . . . .	0 4
Dextrin . . . . .	19.6
Sucrose . . . . .	15 0
Salt mixture‡ . . . . .	4 0
Agar . . . . .	2 0
Lard . . . . .	26 0
Cod liver oil . . . . .	5 0
	100 0

\* The diet contained 21 per cent of "effective" amino acids including glucosamine. The vitamin B factors were supplied in the form of two pills daily, each containing 75 mg. of milk concentrate and 50 mg. of tiki-tiki extract

† For the composition of this mixture, see the paper of Rose (1931).

‡ Osborne and Mendel (1919).

per cent of the preparation of Unknown I or Unknown II, or 2 per cent each of Unknowns I and II. In Table II are recorded the total gains in weight and the total food intakes of the animals. Inasmuch as the preparations were extremely crude, subnormal growth was manifested by all of the animals. However, the gains made by the rats which received both preparations are strikingly better than are the growth accomplishments of the animals which received only one fraction without the other.

## 280      Effect of Feeding Amino Acids.    VII

*These differences provide the first convincing proof of the dual nature of the unknown.*

It occurred to us immediately that one of the factors in question might be a known amino acid which was not present in sufficient amounts in our basal ration. Early in our attempts to induce growth in rats upon mixtures of purified amino acids, we systematically investigated the relation of each of the recognized protein components to the nutritive inadequacy of our standard amino acid mixture (Mixture I (Rose, 1931)). At that time we satisfied ourselves that no single amino acid sufficed to account for the growth failure of our experimental animals. A number of combi-

TABLE II  
*Growth-Stimulating Action of Unknowns I and II*

The experiments covered 24 days

Rat No. and sex	Crude supplement	Total gain in weight	Total food intake
		<i>gm</i>	<i>gm</i>
2193 ♂	4% Unknown I	3	54
2194 ♂		2	43
2195 ♀		0	54
2190 ♂	4% Unknown II	12	66
2191 ♂		15	80
2192 ♀		12	81
2196 ♂	2% Unknown I and 2% Unknown II	34	110
2197 ♂		29	92
2198 ♀		30	101
2199 ♂		36	114

nations, consisting of two or three of the amino acids which we regarded as the most probable limiting factors, were also tested as supplements to our amino acid mixture; but these likewise failed to improve the quality of the diet. For this reason, we did not hesitate to predict the presence in proteins of at least one unrecognized component. On the other hand, it does not follow that when the latter is included in the food, further improvement in quality may not be induced by increasing the proportion of some known amino acid.

After separating the two limiting factors as outlined above, we tested the effect of replacing Unknown I by several amino acids

found to be present in many of our active fractions. It was observed promptly that only isoleucine was capable of inducing growth when Unknown II was included in the ration. *Thus the factor temporarily designated as Unknown I is undoubtedly isoleucine.* A number of growth experiments proving this fact were conducted, but, inasmuch as they were of short duration, they are not reproduced here. Throughout our isolation experiments we have been compelled to rely upon short feeding trials in order to conserve amino acids and to economize in the use of the protein concentrates being tested. However, experience has served to demonstrate that short tests of this sort are thoroughly trustworthy. The response of young rats upon our basal diet to the administration of a missing amino acid is truly remarkable. Frequently, the animals gain several gm. overnight. Such tests are continued ordinarily for 4, 8, or 10 days, depending upon the observed acceleration in growth rate. In the case in question, the inclusion of 1 per cent of isoleucine in the diet of rats receiving amino acid Mixture I and Unknown II, in addition to the other components of a well balanced ration, immediately induced gains in weight of 2 to 3 gm. per day. Similar results were obtained simultaneously by McCoy and Rose (unpublished data).

Finally, an explanation is in order for the unexpected isoleucine deficiency of our amino acid Mixture I. As already pointed out (Rose, 1931), this mixture was formulated to imitate the composition of casein in so far as the best available analyses of this protein permitted. No reliable information is to be found in the literature regarding the isoleucine content of proteins. It is well known, however, that natural leucine contains more or less isoleucine, and perhaps some norleucine. In the case of casein, 9.7 per cent is the generally accepted value for the leucine content, including the unknown quantity of isoleucine. Hence in preparing our amino acid mixture we reduced the quantity of *natural* leucine to 9 per cent, and added 2.5 gm. each of *dl*-isoleucine and *dl*-norleucine. At the time, we stated that these values represented "little more than guesses" (Rose, 1931). Thus, in so far as one could predict, Mixture I provided a sufficient quantity of each of the leucines. Furthermore, experiments showed that additional quantities of isoleucine, without the concentrates of the unknown essential, failed to improve growth; but the addition of crude preparations

of the unknown, without added isoleucine, was followed immediately by increases in the weights of the experimental animals. Under the circumstances, the only way in which an isoleucine deficiency could be demonstrated was by separating the active concentrates into two fractions, and proving that in one isoleucine was the essential component. This has now been accomplished. Failure to recognize earlier the importance of isoleucine as a constituent of the growth-stimulating fractions delayed the identification of Unknown II for at least 2 years. It is an odd fact that in the meantime, by the use of mixtures of purified amino acids supplemented with active concentrates of proteins, we had demonstrated the essential nature of both leucine and isoleucine (Womack and Rose, 1934). These data will be reported in full in a later paper.

#### SUMMARY

Evidence has been presented demonstrating that the "unknown growth essential" referred to in the earlier papers of this series is composed of two factors. These have now been separated by their solubility difference in aqueous butyl alcohol. The more soluble component has been identified as isoleucine. The recognition of this fact has greatly facilitated the isolation and identification of the second growth essential.

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## FEEDING EXPERIMENTS WITH MIXTURES OF HIGHLY PURIFIED AMINO ACIDS

### VIII. ISOLATION AND IDENTIFICATION OF A NEW ESSENTIAL AMINO ACID\*

By RICHARD H. MCCOY,<sup>††</sup> CURTIS E. MEYER,<sup>‡</sup> AND WILLIAM  
C. ROSE

(From the Laboratory of Physiological Chemistry, University of Illinois,  
Urbana)

(Received for publication, August 26, 1935)

Having demonstrated in Paper VII that our so called "growth essential" consists of two components, one of which is isoleucine, we next directed our efforts toward the isolation and identification of the other factor (temporarily denoted as Unknown II). By increasing the isoleucine content of our basal diet we were able to follow the concentration of Unknown II, by determining the growth-stimulating action of each fraction. In doing so no apparent disappearance of activity was observed as purity was approached, such as occurred so frequently in our earlier investigations when inadequate quantities of isoleucine were present in the food.

The experiments led to the isolation of Unknown II in pure form, and its identification. The methods employed and the evidence for the structure of the compound are described below.

\* Aided by a grant from the Graduate School Research Fund of the University of Illinois.

The data in this paper were presented in abstract before the American Society of Biological Chemists at Detroit, April 11, 1935 (Rose, W. C., McCoy, R. H., Meyer, C. E., Carter, H. E., Womack, M., and Mertz, E. T., *J. Biol. Chem.*, 109, lxxvii (1935)).

† Fellow of the General Education Board, New York.

‡ The experimental data in this paper are taken from theses submitted by Richard H. McCoy and Curtis E. Meyer in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiological Chemistry in the Graduate School of the University of Illinois.



## EXPERIMENTAL

*Composition of the Amino Acid Mixture*—At the beginning, it became necessary to modify the composition of the amino acid mixture used in the basal diets. The amount of *dl*-isoleucine was raised from 2.5 gm. to 8.0 gm. At the same time, the proportions of glycine, hydroxyproline, and serine were also increased. No conclusive evidence exists for the indispensable nature of either of these protein components. However, the quantities present in Mixture I were quite low. In order to avoid the possibility of another deficiency involving one or more of these acids, the amount of each was increased. The proportions of the other amino acids were kept at the levels present in Mixture I, with the exception of methionine. The latter was included in the new mixture (Mixture XII) instead of being added separately to the diet as in our former experiments.

The composition of Mixture XII is shown in Table I. For purposes of comparison, the make-up of Mixture I and the supposed composition of casein are presented also. As will be observed, seven of the amino acids in Mixture XII are synthetic products. As in the past, hydroxyglutamic acid is not included. The remaining acids were prepared in this laboratory from their natural sources. All were thoroughly purified and analyzed before being used.

*Isolation of Unknown II*—For the isolation of the growth essential, procedures were employed similar to those which have been used repeatedly in this laboratory for the preparation of active fractions of proteins. The concentrates thus obtained were further purified by treatment with phosphotungstic acid under suitable conditions, and the amino acids from the soluble phosphotungstates were fractionally crystallized from aqueous alcohol solutions.

In the preparation of the pure compound, 12 kilos of commercial fibrin were hydrolyzed with sulfuric acid in the usual manner (Berg and Rose, 1929). After removal of the sulfuric acid the solution was concentrated *in vacuo*, and cooled. The less soluble amino acids which separated (2650 gm.) were filtered off. The amino acids in the filtrate were converted to their copper salts by heating with an excess of basic copper carbonate, and the solution was concentrated *in vacuo*. Several fractions of the less

TABLE I  
Composition of Amino Acid Mixtures

	Casein	Mixture I		Mixture XII	
		Active amino acids	As used	Active amino acids	As used
	gm	gm	gm	gm	gm
Glycine	0 45	0 50	0 50	3 00	3 00
Alanine.	1 85	1 90	3 80*	1 90	3 80*
Valine . . . . .	7 93	8 00	16 00*	8 00	16 00*
Leucine ..	9 70†	9 00	9 00	9 00	9 00
Isoleucine . . . . .	?	1 25	2 50*	4 00	8 00*
Norleucine . . . . .	?	1 25	2 50*	1 25	2 50*
Proline . . . . .	7 63	8 00	8 00	8 00	8 00
Hydroxyproline	0 23	0 30	0 30	2 00	2 00
Phenylalanine . . . . .	3 88	3 90	7 80*	3 90	7 80*
Glutamic acid	21 77	22 00	22 00	22 00	22 00
Hydroxyglutamic acid	10 50	0	0	0	0
Aspartic acid	4 10	4.10	4 10	4 10	4 10
Serine . . . . .	0 50	0 50	1 00*	1.50	3 00*
Tyrosine . . . . .	4 50	6 50	6 50	6.50	6 50
Cystine ..	?	1 25	1 25	1 25	1 25
Histidine . . . . .	2 50	2 75		2 75	
“ hydrochloride			3 40		3 40
Arginine . . . . .	3 81	5 25		5 25	
“ hydrochloride			6 35		6 35
Lysine ..	7 62	7 70		7 70	
“ dihydrochloride.			11 55		11 55
Tryptophane	1 50	2 25	2 25	2 25	2 25
Methionine . . . . .	3 50‡	§		1 75	3 50*
Sodium bicarbonate .. . . .			12 86		12 86
	91 97	86 40	121 66	96 10	136 86¶

\* Racemic acids

† Includes isoleucine.

‡ Baernstein (1934).

§ 0.6 per cent *DL*-methionine added directly to the diet.

|| 1 408 gm. of Mixture I are equivalent to 1.0 gm of “effective” amino acids.

¶ 1.424 gm of Mixture XII are equivalent to 1.0 gm. of “effective” amino acids.

soluble copper salts (totaling 1365 gm.) were removed. From the filtrate the copper was precipitated with hydrogen sulfide, and the resulting solution was concentrated *in vacuo* to a volume of 7 liters.

The above solution was extracted seventeen times with 40 liter portions of butyl alcohol, as described in Paper VII. Each extract was concentrated to approximately one-third of its original volume, cooled for 12 hours, and filtered. The amino acids recovered from each extract were washed several times with dry butyl alcohol. Extracts 1 to 3 inclusive (246 gm.) were discarded. Extract 14 was employed as a crude source of Unknown II in certain preliminary tests. The remaining material (Extracts 4 to 13 and 15 to 17 inclusive), amounting to 324 gm., was suspended in 1300 cc. of water and thoroughly mixed. 5.8 gm. which failed to dissolve were filtered off and discarded. The solution was then reextracted three times with 1.5 liter portions of butyl alcohol, and once with 5 liters of the solvent. The combined material recovered from the four extracts amounted to 56.4 gm. Growth tests showed that it was practically devoid of activity; hence it was discarded. The amino acids in the aqueous solution amounted to about 262 gm. (by difference).

At this point, a large part of the aqueous solution was used in a number of unsuccessful attempts to effect a concentration of the unknown. Inasmuch as the measures employed were not satisfactory, they need not be described.

Of the remaining amino acids from the aqueous solution, 123 gm. were dissolved in 500 cc. of 5 per cent sulfuric acid, and treated with a large excess of phosphotungstic acid in 5 per cent sulfuric acid. The mixture was allowed to stand at room temperature for 18 hours and filtered. The precipitate was washed thoroughly with a cold solution of phosphotungstic acid in 5 per cent sulfuric acid. The combined filtrate and washings were evaporated *in vacuo* until the sulfuric acid had attained a concentration of approximately 12 per cent. After standing at 0° for 48 hours a very granular precipitate (86 gm.), containing no active material, was removed by filtration and discarded. Levene and Van Slyke (1913-14) have shown that the phosphotungstates of glycine, alanine, and valine are only moderately soluble under these conditions. Their observation provided a means of removing these amino acids which, with isoleucine, have been recognized for some time as the chief contaminants of our growth essential.

From the filtrate, the phosphotungstic and sulfuric acids were removed by the addition of an excess of barium hydroxide. The

precipitate was thoroughly washed, and the filtrate and washings were freed of barium by the cautious addition of sulfuric acid. The almost colorless solution was concentrated *in vacuo* to a volume of about 175 cc., and placed in a refrigerator overnight. 8.6 gm. of crystalline material separated, which showed practically no growth-stimulating effect. The filtrate was then subjected to repeated fractional precipitations with ethyl alcohol. These need not be described in detail. The number of precipitations and recrystallizations necessary depends upon the degree of purity of the growth essential when the alcohol treatments are begun. It is noteworthy, however, that the first sign of approaching purity of the compound is the separation of microscopic needle-shaped crystals, usually in rosettes. They are somewhat like tyrosine in appearance (Fig. 1), but do not respond to tyrosine tests. After a number of additional recrystallizations, the pure compound gradually appears in the form of beautiful hexagonal plates (Fig. 2). These crystals, which are not unlike those of serine, are characteristic of the new growth essential. In our experience, only the hexagonal crystals give correct results on analysis, or manifest the maximum nutritional action. As the purity of the compound increases, the concentration of alcohol necessary to induce crystallization diminishes. A concentration of about 40 volumes per cent is quite satisfactory for the later crystallizations.

The yield of the pure compound obtained by the above method, after all of the mother liquors had been reworked, amounted to 4.5 gm. from the 123 gm. of crude material subjected to the phosphotungstic acid treatment. This is equivalent to about 0.8 gm. per kilo of fibrin.

A second isolation of the growth essential was brought to a successful conclusion a few days after the first. Since then the compound has been prepared a number of times by various modifications of the general procedure outlined above. Some of these methods will be discussed in later papers. However, no procedure has been found which is not exceedingly laborious, and which does not involve a considerable number of recrystallizations after the substance has been freed of a large proportion of its contaminants. Apparently, the compound is intimately associated with some other material which is very difficult to remove. Furthermore, our yields undoubtedly represent a very small part of the amount

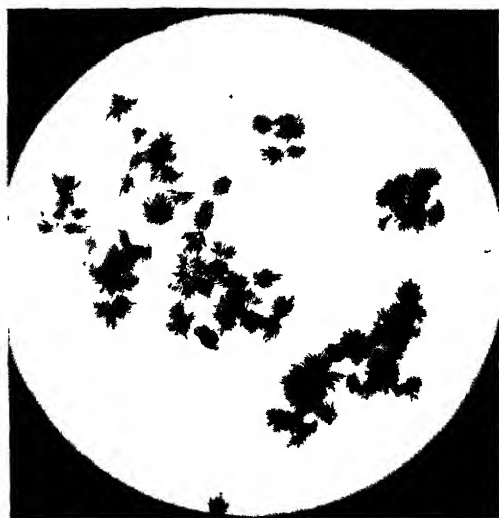


FIG 1 Impure amino acid (140 X)

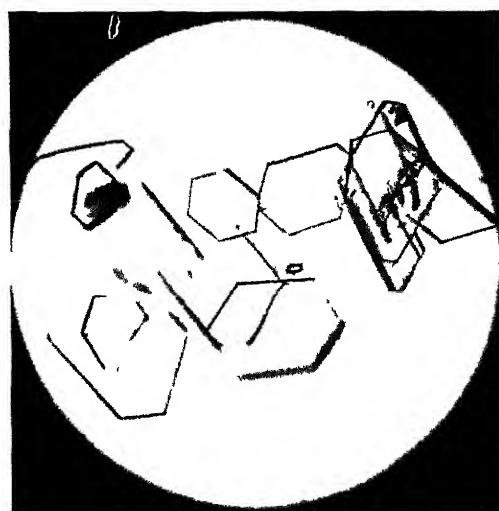


FIG 2. Pure amino acid (140 X)

actually present in proteins. Possibly an 80 to 90 per cent loss may occur during the isolation. Efforts are now being made to improve the procedure, with the expectation that within a few months a more abundant supply of the compound may be available

*Growth Effect of the Pure Compound*—Throughout the process of isolation, the various fractions were tested for their growth-stimulating action. When the hexagonal plates were obtained, and shown to be of constant composition, it became necessary to demonstrate that they exerted the expected physiological effect. For this purpose, the compound was incorporated in the food of rats, first at levels of 0.4 and 0.5 per cent respectively. After 16 days, the amount of the supplement was increased in all animals, except the controls, to 0.6 per cent. These varying proportions were employed in order to determine the approximate quantity of the substance necessary to induce maximum growth

The composition of the diets is shown in Table II. Diet 40 was devoid of the new essential. Diets 41, 42, and 43 carried the supplement in the amounts indicated. Each ration furnished 18 per cent of "effective" amino acids including glucosamine. The vitamin B factors were supplied in the form of two pills daily, each containing 50 mg. of tikitiki extract and 75 mg. of milk concentrate. The daily intake of nitrogen from these sources amounted to approximately 4 mg., and was the only nitrogen of unknown kind.

The results of the feeding trials are shown in Chart I. In Table III are recorded the total change in weight and total food intake of each rat. *The data demonstrate conclusively that the crystalline compound is the new essential we have been endeavoring to isolate for several years. Furthermore, the experiments recorded in Chart I represent the first successful efforts to induce growth in animals upon diets carrying synthetic mixtures of highly purified amino acids in place of proteins.* Because of the necessity of conserving our limited supply of the new essential for studies of its chemical structure, few animals were employed in the growth studies. However, the results are so clear cut as to leave no doubt as to their significance. Furthermore, the data presented in Chart I have been confirmed repeatedly in connection with other investigations, the results of which will be presented in subsequent papers.

It is evident from Chart I that 0.4 per cent of the new essential

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did not permit as rapid increase in weight as did 0.5 per cent. When the quantity was raised to 0.6 per cent, the animals receiving the lower level showed a pronounced improvement in growth. On the other hand, those receiving 0.5 per cent manifested very little growth acceleration. Evidently, 0.6 per cent is approximately the minimum amount which must be in the food in order to induce maximum growth in rats. It is to be noted also that *hydroxyglu-*

TABLE II  
*Composition of Diets\**

	Diet 40	Diet 41	Diet 42	Diet 43
	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
Amino acid Mixture XII	24.45	23.88	23.74	23.60
Glucosamine hydrochloride ( <i>d</i> -)	1.00	1.00	1.00	1.00
Sodium bicarbonate	0.40	0.40	0.40	0.40
Dextrin	22.15	22.32	22.36	22.40
Sucrose	15.00	15.00	15.00	15.00
Salt mixture†	4.00	4.00	4.00	4.00
Agar. . . . .	2.00	2.00	2.00	2.00
Lard	26.00	26.00	26.00	26.00
Cod liver oil . . . . .	5.00	5.00	5.00	5.00
New growth essential	0	0.40	0.50	0.60
	100.00	100.00	100.00	100.00

\* Each diet contained 18 per cent of "effective" amino acids, including glucosamine. The vitamin B factors were supplied in the form of two pills daily, each containing 50 mg. of tikitiki extract and 75 mg. of milk concentrate. The daily intake of nitrogen from these sources amounted to approximately 4 mg.

† Osborne and Mendel (1919)

*tamic acid and citrulline are dispensable components of the food, inasmuch as neither of these amino acids was available to the animals.*

*Physical and Chemical Properties of the New Essential*—In Table IV are summarized the results of analyses of several different preparations of the compound. The specific rotation of each is also given. As will be observed, the analytical data agree with the calculated values for a compound having the empirical formula,  $C_4H_9NO_3$ . Previously, McCoy and Rose (*cf.* Caldwell and Rose (1934), p. 71) have shown that the ethyl and butyl esters of the growth essential can be distilled, but without increase in the

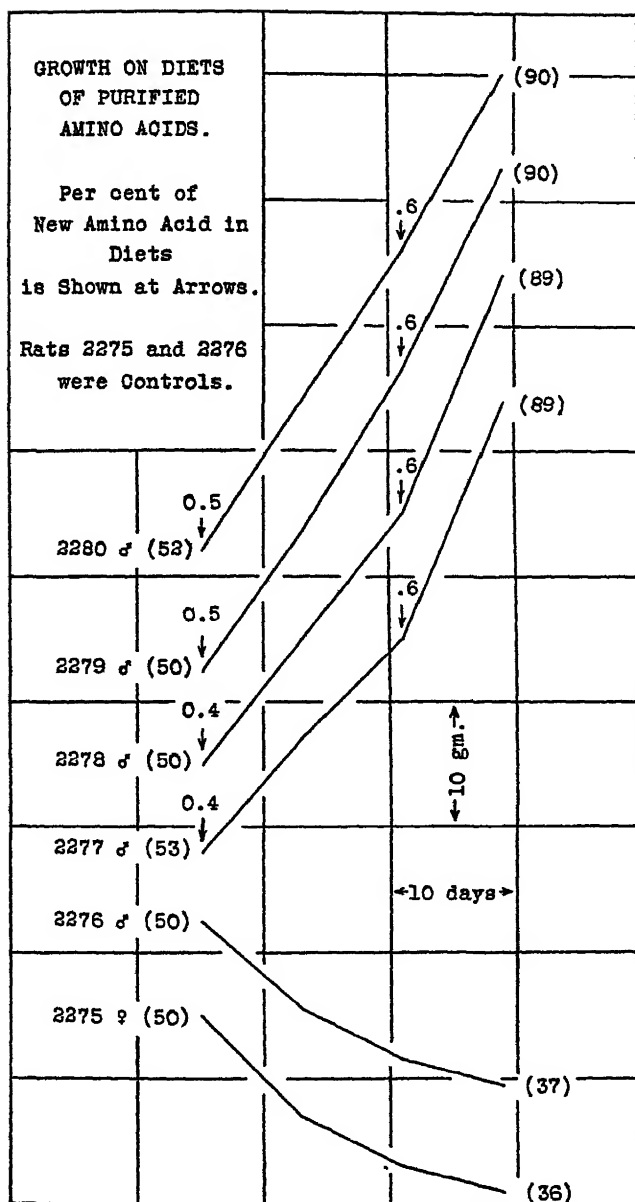


CHART I The numbers in parentheses denote the initial and final weights of the rats.



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potency of the fractions, and that treatment of concentrates with nitrous acid destroys the growth-stimulating action. These facts

TABLE III  
*Total Changes in Body Weight and Total Food Intakes of Animals on Diets Containing Purified Amino Acids*

Rat No and sex	$\alpha$ -Amino- $\beta$ -hydroxybutyric acid	Days	Total change in weight	Total food intake
	<i>per cent</i>		<i>gm</i>	<i>gm</i>
2280♂	0 5	16	+24	64
	0 6	8	+14	34
2279♂	0 5	16	+24	66
	0 6	8	+16	38
2278♂	0 4	16	+20	65
	0 6	8	+19	38
2277♂	0 4	16	+17	65
	0 6	8	+19	37
2276♂	Control	24	-13	38
2275♀	"	24	-14	39

TABLE IV  
*Analyses of New Growth Essential*

Preparation No	C	H	N	O (by difference)	$[\alpha]_D$	<i>t</i>
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>degrees</i>	<i>°C</i>
1*	40 50	7 59	11 76	40 15	-27 53	25
2	40 60	7 65	11 67	40 08	-27 50	25
3	40 41	7 50	11 78	40 31	-28 23	25
4	40 18	7 66	11 71	40 45	-27 51	27
5			11 80		-27 83	25
6			11 84		-28 03	22
Calculated for $C_4H_9NO_3$	40 31	7 62	11 76	40 31		

\* This product was isolated in March, 1934. It was not recognized as the growth essential because of the isoleucine deficiency in our basal diet. Its inclusion in the food failed to improve growth until amino acid Mixture XII was substituted for Mixture I almost a year later.

pointed to the probability that the compound was one of the possible hydroxyaminobutyric acids. This was further indicated by a determination of the molecular weight of the crystalline mate-

rial in aqueous solution by the cryoscopic method. The result was as follows:

$C_4H_8NO_2$  Theoretical mol. wt, 119.1; found, 117.3

The compound gives a strong ninhydrin reaction, and responds to the biuret test, as do several hydroxyamino acids, including serine. By the Van Slyke method all preparations yield high values for amino nitrogen. Determination of the melting point showed decomposition over a wide range of temperatures. Darkening usually begins at about  $210^\circ$ , and the substance melts with decomposition at about  $255\text{--}257^\circ$ .

Several derivatives were prepared as outlined below.

*Copper Salt*—0.45 gm. of the pure crystalline material was dissolved in 8 cc. of water and treated with an excess of basic copper carbonate. The solution was boiled for a short time and filtered. The filtrate was evaporated to a small volume and again filtered. The resulting solution was placed in a vacuum desiccator over phosphorus pentoxide. In a short time beautiful rectangular plates appeared, which were removed by filtration and washed with a small amount of cold water. The crystals were ground in an agate mortar, and dried thoroughly in an Abderhalden dryer. Analysis yielded the following values.

$(C_4H_8NO_2)_2Cu$  Calculated. C 32.03, H 5.38, N 9.35, Cu 21.21  
Found. " 31.91, " 5.45, " 9.42, " 20.87

Theoretical mol. wt, 299.7; calculated from Cu content, 304.6  
Theoretical equivalent weight, 118.1; calculated from Cu content, 120.0

*Benzoyl Derivative*—The N-benzoyl derivative was prepared according to the procedure of Sorensen and Andersen (1908). For this purpose, 1 gm. of the amino acid was dissolved in 5.7 cc. of water and 4.32 cc. of 3.12 N sodium hydroxide. 4 cc. of benzoyl chloride and 22 cc. of 3.12 N sodium hydroxide were added in ten equal portions at intervals of 5 to 10 minutes. The solution was kept cold during these additions. When the reaction was complete, the solution was acidified with 10 cc. of dilute hydrochloric acid, and filtered for the removal of the precipitated benzoic acid. The filtrate was evaporated to dryness *in vacuo*, and the residue was taken up in water and again evaporated to dryness. This process was repeated twice with absolute alcohol instead of water. The



butyric acids. It is obvious that twenty such compounds may exist if one takes into consideration both the structural and optical isomers. In determining the constitution of the acid, the position of the amino group was first established by reducing the hydroxy-amino acid to the corresponding amino acid. The reduction was carried out by a procedure similar to that employed by Abderhalden and Heyns (1934). For this purpose, a mixture of 1 gm. of the hydroxyamino acid, 0.3 gm. of red phosphorus, and 12 cc. of hydriodic acid (sp. gr. 1.96) was heated in a sealed tube at 160° for 5½ hours. After cooling, the contents of the tube were diluted with about 300 cc. of water, and evaporated to dryness *in vacuo*. The residue was taken up in water, and treated with an excess of moist silver oxide. The silver iodide and excess silver oxide were filtered off. The filtrate was treated with hydrogen sulfide for the removal of the last of the silver ions, and after being filtered again, was concentrated *in vacuo* to a volume of about 5 cc. On diluting to 12 cc. and adding 17 cc. of absolute alcohol, a crystalline precipitate formed and was filtered off. The latter was recrystallized from 3 cc. of water and 2 cc. of absolute alcohol. The crystals consisted of thin hexagonal plates having the following composition.

$C_4H_9NO_2$ .	Calculated	C 46.57, H 8.80, N 13.59
	Found.	" 46.65, " 8.62, " 13.53

When the crystals were dissolved in water, the solution showed a slight but unmistakable *dextrorotation*, in contrast to the unreduced acid which manifests a strong *levorotation*. Unfortunately, it was not possible to determine accurately the specific rotation of the reduced product because of the small amount of material available. It is of importance to note, however, that according to Oikawa (1925) naturally occurring  $\alpha$ -amino-*n*-butyric acid has a specific rotation of +8.05°. Fischer and Mouneyrat (1900) report specific rotations of +8.0° and -7.92° respectively for the two enantiomorphs prepared by the resolution of synthetic  $\alpha$ -aminobutyric acid. Evidently, considerable racemization occurred during the reduction of our hydroxyamino acid, but the inversion of the direction of rotation is quite significant.

The melting point of the reduced material varied from 277-304°, depending upon the speed with which the bath was heated. For

purposes of comparison, three products of entirely different origin were melted simultaneously in the same bath with rather rapid heating. These were (a) the reduction product prepared from the natural hydroxyaminobutyric acid, (b) *dl*- $\alpha$ -amino-*n*-butyric acid (Eastman), and (c) a product obtained by reducing synthetic  $\alpha$ -amino- $\beta$ -hydroxybutyric acid. In each case the compound melted with decomposition. The results are tabulated below.

	°C
Reduction product from natural acid . . . .	297 (Corrected)
<i>dl</i> - $\alpha$ -Aminobutyric acid (Eastman) . . . .	299    "
Reduction product from synthetic $\alpha$ -amino- $\beta$ -hydroxybutyric acid . . . . .	298    "

In a similar fashion the melting points of our reduction product and  $\alpha$ -aminoisobutyric acid (Eastman) were compared simultaneously. With slow heating in the same bath the following values were obtained.

	°C
Reduction product from natural acid . . . .	280 (Corrected)
$\alpha$ -Aminoisobutyric acid . . . . .	319-320    "

Obviously, the reduced material is not identical with  $\alpha$ -aminobutyric acid. The latter is also excluded by the fact that it is inactive toward polarized light. The other possible products which might have been obtained by the reduction of the new amino acid are  $\beta$ -amino-*n*-butyric acid,  $\gamma$ -amino-*n*-butyric acid, and  $\beta$ -aminoisobutyric acid. The first of these is excluded, inasmuch as the synthetic compound melts at 191-192° (Abderhalden, 1924).  $\gamma$ -Amino-*n*-butyric acid melts at 203° (Abderhalden, 1924), and in contrast to our reduced material is inactive toward polarized light. Apparently, no information is recorded in the literature regarding the melting point of  $\beta$ -aminoisobutyric acid. According to Abderhalden and Heyns (1934),  $\beta$ -amino acids do not give the ninhydrin reaction. We have confirmed Abderhalden's statement with respect to one such acid, namely,  $\beta$ -amino-*n*-valeric acid.<sup>1</sup> Our reduction product, however, gives an intense response to the ninhydrin test. In any event, the identical melting points, within the limits of experimental error, of our reduced product and

<sup>1</sup> We are indebted to Dr. H. D. Dakin for the sample of  $\beta$ -aminovaleric acid.

synthetic  $\alpha$ -aminobutyric acid leave no doubt that the two compounds are the same. Furthermore, Oikawa (1925) reports a melting point of  $301^{\circ}$  (closed tube) for his natural *d*- $\alpha$ -aminobutyric acid. Thus the spatial configuration of the groups around the  $\alpha$ -carbon atom of our hydroxyaminobutyric acid is probably identical with that which exists in natural  $\alpha$ -aminobutyric acid.

Having shown that the hydroxyaminobutyric acid of proteins yields *d*- $\alpha$ -amino-*n*-butyric acid on reduction, the position of the hydroxyl group obviously is limited to either the  $\beta$  or  $\gamma$  position. Fischer and Blumenthal (1907) and Sorensen and Andersen (1908) have synthesized  $\alpha$ -amino- $\gamma$ -hydroxy-*n*-butyric acid. The latter investigators found that the monobenzoyl derivative of this compound readily yields the corresponding lactone, as would be expected, when warmed in acid solution. Under similar conditions, the monobenzoyl derivative of our hydroxyaminobutyric acid does not yield a lactone. This would appear to exclude the possibility of the hydroxyl group in our amino acid being in the  $\gamma$  position. In order to verify this conclusion,  $\alpha$ -amino- $\gamma$ -hydroxy-*n*-butyric acid was synthesized by a modification of the method of Fischer and Blumenthal (1907), and tested for its growth effect upon animals.

The synthesis was carried out as follows: 248 gm. of methylcellosolve were dissolved in 615 gm. of diethylaniline in a three-necked flask equipped with a mechanical stirrer and dropping funnel. Through the latter, 570 gm. of thionyl chloride dissolved in 400 cc. of chloroform were added slowly, the temperature of the mixture being kept below  $25^{\circ}$ . The whole was allowed to stand at room temperature overnight, and then heated for 30 minutes on a steam bath. After cooling, the solution was poured into 2 liters of dilute hydrochloric acid containing crushed ice. The chloroform and aqueous layer were separated, and the latter was extracted twice with additional portions of chloroform. The combined chloroform solutions were washed three times with dilute hydrochloric acid for the removal of the diethylaniline, and finally with a very dilute solution of sodium bicarbonate. The chloroform was separated from the methoxyethyl chloride by fractional distillation. 164.6 gm. of the latter were obtained, having a boiling point of  $89-90^{\circ}$ .

The methoxyethyl chloride was condensed with sodium malonic

ester according to the procedure of Palomaa and Kenetti (1931). The yield of methoxyethyl malonic ester amounted to 32 per cent of the theory. The density of the ester at 20° was 1.0395 as compared with 1.0397 reported by Palomaa and Kenetti. The refractive index was  $n_D^{20} = 1.4258$ .

The ester was saponified by refluxing for 10 hours with 10 volumes of 95 per cent alcohol containing 4 moles of potassium hydroxide. The alcohol was removed by evaporation on a steam bath, and the residue was dissolved in iced, dilute hydrochloric acid. The methoxyethyl malonic acid was removed by extraction with 20 portions of ether, the ether was evaporated, and the residue was dried in a vacuum desiccator over phosphorus pentoxide. The solid was then dissolved in 600 cc. of dry ether, and treated with 80 gm. of bromine added dropwise with stirring. The flask was cooled occasionally as required. When the reaction was complete, the excess bromine was removed by shaking the contents of the flask with a dilute solution of sodium bisulfite. The ethereal solution was dried with anhydrous sodium sulfate, and transferred to a distillation flask. After the ether had been removed, the temperature was raised to 150° in order to decompose the substituted malonic acid. This was accomplished in about 10 minutes. The  $\alpha$ -bromo- $\gamma$ -methoxybutyric acid was distilled *in vacuo*. It boiled between 145–148° at a pressure of 5 mm. The neutral equivalent of the acid was found to be 200 as compared with a calculated value of 197.

For the formation of the methoxyamino acid 20 gm. of the methoxybromo acid were heated for 6 hours in a pressure flask with 100 cc. of concentrated ammonium hydroxide. The resulting solution was reduced to dryness *in vacuo*. The methoxyamino acid was separated from the ammonium bromide by dissolving the mixture in 20 cc. of water, and adding alcohol to incipient crystallization. The acid was recrystallized in the same manner. The yield in this step was 62 per cent of the theory. The compound melted with decomposition at 224–225°. Analysis gave the following result.

$C_8H_{11}NO_3$     Calculated, N 10.52; found, N 10.42

The methoxy group was hydrolyzed by refluxing the above compound for 3 hours with 48 per cent hydrobromic acid. The

resulting solution was reduced to dryness *in vacuo*. The residue was dissolved in 100 cc. of water and treated with an excess of moist silver oxide. After filtering, the remaining silver was removed with hydrogen sulfide. The filtrate was evaporated *in vacuo*, yielding an alkaline syrup of the lactone. The lactone was dissolved in 50 cc. of water and rapidly evaporated on a steam bath. During the concentration the reaction of the solution changed from alkaline to slightly acid, indicating the opening of the lactone ring. Addition of alcohol resulted in the crystallization of the  $\alpha$ -amino- $\gamma$ -hydroxybutyric acid. Recrystallization was accomplished by dissolving the amino acid in the minimum quantity of hot water, cooling, and adding alcohol. Analysis of the crystalline compound gave the following nitrogen content

$C_4H_7NO_3$ . Calculated, N 11.76; found, N 11.63

When this compound was incorporated at a level of 4 per cent, in an otherwise adequate diet, it exerted absolutely no growth-stimulating effect. It is certain, therefore, that the hydroxyaminobutyric acid isolated from proteins is not  $\alpha$ -amino- $\gamma$ -hydroxybutyric acid.

*From the investigations outlined above it follows inevitably that the new amino acid is one of the four optically active  $\alpha$ -amino- $\beta$ -hydroxy-*n*-butyric acids. Inasmuch as the reduction product derived from the natural amino acid is *d*- $\alpha$ -amino-*n*-butyric acid, the only structural detail yet unsolved is the spatial configuration around the  $\beta$ -carbon atom. This is now being investigated, and the results will be presented in a subsequent paper of this series.*

As confirmatory evidence for the general nature of the compound, Dr. H. E. Carter of this laboratory has accomplished the synthesis of a mixture of the four optically isomeric  $\alpha$ -amino- $\beta$ -hydroxybutyric acids. The pair of mirror image products synthesized from crotonic acid exerts extremely little if any growth stimulation. After epimerization of this pair, the resulting material manifests about one-fourth to one-sixth of the activity of the natural amino acid. This synthesis will be published in detail in the near future.

*Previous Reports of the Isolation of Hydroxyaminobutyric Acid—*The possible isolation of hydroxyaminobutyric acid from proteins has been reported previously by three groups of investigators. Of



these Gortner and Hoffman (1925) were the first. Their product contained 28 per cent of ash. After correcting for this enormous amount of inorganic material, the elementary analysis showed C 39.93, H 9.09, and N 12.00 per cent, indicating "an empirical formula of  $C_4H_{11}O_3N$  or some multiple of this." That the compound may have been hydroxyaminobutyric acid is mentioned by the authors merely as a possibility. Unfortunately, the empirical formula given by them is not that of hydroxyaminobutyric acid, nor do the analytical data correspond satisfactorily to the theoretical values for  $C_4H_9NO_3$ .

Schryver and Buston (1925-26) reported the isolation of an amino acid which they believed to be hydroxyaminobutyric acid. In separating it from the other amino acids they made use of the solubilities of its zinc and copper salts. According to these investigators, the zinc salt of the amino acid is soluble in ethyl alcohol; but the copper salt is insoluble in dry methyl alcohol, even when the extractions are continued till the extract is no longer blue. Invariably, we have found our compound in the fraction of amino acids whose copper salts are soluble in absolute methyl alcohol. Admittedly, the solubility is slight, as was pointed out by Caldwell and Rose (1934), but appreciable quantities continue to dissolve almost indefinitely during repeated extractions. Furthermore, the zinc salt of our amino acid dissolves very slowly in ethyl alcohol of the strength employed by Schryver and Buston. Because of these apparent differences in properties we concluded 2 years ago that our growth essential could not be identical with the compound of Schryver and Buston (*cf.* Caldwell and Rose (1934)). Despite these discrepancies, there seems to be little reason to doubt that the British investigators really had hydroxyaminobutyric acid. Their analyses agree well with the values required by the formula. They report also the preparation of the dibenzoyl and phenylisocyanate derivatives, each of which analyzed satisfactorily. However, they record no attempts to determine the structure or physiological importance of the amino acid.

Shortly after the work of Schryver and Buston, Rimington (1927) described the isolation of hydroxyaminobutyric acid from phosphopeptone. With the exception of the nitrogen value, the reported analytical data are not in close agreement with those required by a compound having an empirical formula,  $C_4H_9NO_3$ .

Although no evidence is presented for the structure of the acid, its formula is written by the author as  $\alpha$ -amino- $\beta$ -hydroxybutyric acid

In conclusion, attention may be called to the fact that the isolation of  $\alpha$ -amino- $\beta$ -hydroxybutyric acid, and the proof that it is the hitherto unknown growth essential, renders it possible for the first time, to rear animals on diets containing mixtures of highly purified amino acids in place of proteins. By means of such experiments the physiological significance of the amino acids may be established by dropping them out of the diet one at a time. Several such investigations are already under way in this laboratory. It is our purpose also to determine the *quantitative* needs of the organism for each nitrogenous essential. Eventually, such data may provide a new and final answer to the question of the protein requirements of man and animals

#### SUMMARY

The hitherto unknown growth essential has been isolated in pure, crystalline form, and identified as one of the four optically isomeric  $\alpha$ -amino- $\beta$ -hydroxy-*n*-butyric acids. Several of its derivatives have been prepared and described.

On reduction the new amino acid yields *d*- $\alpha$ -amino-*n*-butyric acid. The spatial configuration around the  $\beta$ -carbon atom remains to be determined, and is now being investigated.

With an otherwise adequate diet, approximately 0.6 per cent of  $\alpha$ -amino- $\beta$ -hydroxybutyric acid is the minimum amount necessary to induce maximum growth. The feeding trials herein described constitute the first successful attempt to rear animals on a ration containing purified amino acids as the sole source of nitrogen.

Inasmuch as satisfactory growth has been secured by the use of diets devoid of hydroxyglutamic acid and citrulline, these two amino acids are not indispensable components of the food. Experiments designed to determine the physiological importance of the remaining amino acids are now under way.

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# THE EFFECT OF FLUORINE UPON THE PHOSPHATASE CONTENT OF PLASMA, BONES, AND TEETH OF ALBINO RATS

BY MARGARET CAMMACK SMITH AND EDITH M. LANTZ

*(From the Department of Nutrition, Agricultural Experiment Station, the  
University of Arizona, Tucson)*

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In 1923 Robinson (1) demonstrated the presence in ossifying bone of a phosphoric acid ester-splitting enzyme which he believed to play an important rôle in bone calcification. Kay (2) and Bodansky and Jaffe (3) have since made extensive studies of plasma and serum phosphatase under various conditions. In general it appears from their work that plasma phosphatase is changed from the normal in many conditions in which calcium metabolism is disturbed. Kay reports that the phosphatase value of plasma in many cases of generalized bone disease rises to more than 20 times the normal value, the degree of increase being correlated with the severity of the disease.

Work in this laboratory has shown that fluorine interferes with the normal development of teeth and bones. In 1932 (4) fluorine in drinking water was shown to be the cause of a dental disease in humans known as mottled enamel, and this dental damage was produced experimentally (5) in rats, dogs, and guinea pigs by fluorine feeding or injections. The extensive investigation of the effect of chronic fluorine poisoning upon the metabolism of calcium and phosphorus which followed showed that growing fluorine-fed rats (6) and dogs (7) retained less of these mineral elements than do their non-fluorine-fed litter mate controls and the path of excretion was altered. The evidence indicated that fluorine decreased the absorption of calcium.

The possibility of a change in plasma phosphatase values accompanying fluorine toxicosis was suggested by Phillips (8). He briefly reported that the plasma phosphatase of dairy cows fed a

high concentration of fluorine in their ration was practically double that of the control animals. His statement that "the plasma phosphatase in fluorosis forms a sensitive test for the toxic effects of chronic fluorine poisoning" was of great interest to us from the standpoint of a possible application and use in detecting fluorosis in human beings drinking fluorine-containing water and in establishment of the toxic level for fluorine intake.

Our preliminary analyses of the plasma phosphatase in rats suffering from chronic fluorosis did not, however, reveal a higher content than in the control animals. Accordingly, a more thorough investigation of plasma phosphatase of rats in different stages and degrees of fluorine poisoning was conducted.

It has been our observation that a relatively low intake of fluorine by rats (6) and humans (9) results in no measurable interference with calcium metabolism as determined by balance experiments and yet causes a devastating effect upon the enamel of the teeth when no other symptoms of fluorosis are present. Histological examinations (10) of the teeth suggest that fluorine exerts a direct local action on the enamel-forming cells. In order to discover a possible effect of fluorine upon the phosphatase enzymes present at the site of tooth and bone calcification our studies were extended to include phosphatase values of both teeth and bone in fluorine poisoning.

#### EXPERIMENTAL

In the first series of experiments, a study is made of the effect of chronic fluorine intoxication produced by feeding harmful amounts of sodium fluoride upon the phosphatase content of plasma, teeth, and bones of growing rats of different ages. In the second series of experiments, an acute fluorine poisoning is produced by the subcutaneous injection of a solution of sodium fluoride in adult animals and determination of their plasma phosphatase

*Series I*—Litter mate albino rats were divided at weaning (28 days of age) into two groups. One group was given Sherman's Diet B composed of  $\frac{2}{3}$  whole wheat,  $\frac{1}{3}$  whole milk powder, and salt equal to 2 per cent of the weight of the wheat (Diet 13) and was used as a control group. The second group was given the same ration to which 0.1 per cent sodium fluoride had been added. This

ration (Diet 214) has been used in much of the experimental work in this laboratory. It produces severe stunting in growth of the experimental animals, gross changes in tooth and bone structure, and diminished retention of calcium and phosphorus (5, 6, 11). In a few cases, a third group was given the control ration to which 0.025 per cent sodium fluoride had been added. This ration (Diet 216) produces visible changes in the incisor teeth of rats but has little effect on the growth or general health of the animals. Only male rats were used, so as to avoid possible sex differences in phosphatase values.

At 2 week intervals, litter mate rats were taken from each group and the phosphatase content of the plasma and, in most cases, of the bones and teeth was determined.

A fasting blood sample for analysis was taken directly from the heart of the animals which were first anesthetized with chloroform. The animals were deprived of food overnight, because it had been found that extremely variable and much higher results were obtained when a fasting sample was not taken. Phosphatase was determined at first by the method of Kay (12). This method was discarded in favor of the clinical method of Jenner and Kay (13), which can be carried out with smaller blood samples and in a shorter time.

The method consists essentially of incubation of the plasma with the substrate, sodium  $\beta$ -glycerophosphate, highly buffered with a glycine-NaCl-NaOH mixture, for 3 hours at 38°. After precipitation of the proteins with trichloroacetic acid the degree of hydrolysis of the glycerophosphate is estimated by determination of the phosphorus colorimetrically, with stannous chloride as the reducing agent. The results are compared with the results of the non-incubated aliquots and the phosphatase content of the plasmas expressed in terms of the number of mg. of phosphorus which were liberated by 100 cc. of the plasma under the conditions described.

While the blood samples were in the water bath, the incisor teeth and leg bones (tibia) were removed, freed from flesh, and quickly weighed. They were then ground with sand in a mortar and extracted overnight with distilled water to which a few drops of chloroform had been added. The amount of water used was based on the weight of material to be extracted and was 50 times the weight of the bone and 100 times the weight of the teeth

After extraction the mixture was filtered and 0.5 cc. aliquots were taken for analysis by the same method used for blood. The results are expressed as mg. of phosphorus liberated per gm. of fresh tooth or bone material. To exclude the possibility of litter variation or changes in temperature conditions during the determination of phosphatase, comparisons were always made of analyses which were made at the same time on litter mates.

TABLE I

*Effect of Sodium Fluoride on Phosphatase Content of Plasma, Bones, and Teeth of Growing Rats*

No. of litter mate rats on each diet	Length of time on diet after weaning	Age of rats	Average phosphatase content								
			Plasma, mg P liberated per 100 cc plasma			Bones, mg P liberated per gm fresh bone			Teeth, mg P liberated per gm fresh incisor tooth		
			Diet 13 (control)	Diet 214 (0.1 per cent NaF)	Diet 216 (0.025 per cent NaF)	Diet 13 (control)	Diet 214 (0.1 per cent NaF)	Diet 216 (0.025 per cent NaF)	Diet 13 (control)	Diet 214 (0.1 per cent NaF)	Diet 216 (0.025 per cent NaF)
	<i>wks.</i>	<i>wks.</i>									
9	2	6	46.5	31.9	45.6	17.0	16.5	15.6	14.7	14.4	15.4
10	4	8	35.1	31.8	35.7	13.3	13.8	13.3*	11.2	8.4	11.3*
10	6	10	34.8	32.9		10.0	14.4	14.9†	9.5	6.9	9.5†
5	8	12	16.8	20.9		8.4	11.9		16.8	5.4	
2	10	14	13.7	18.4		7.9	13.3		14.0	8.0	
1	11	15	11.0	17.2							
1	12	16	15.1	16.8		10.0	10.6		15.6	4.2	
2	18	22	15.8	15.8		8.3	6.8		10.3		
1	30	34	14.1	17.2		3.9	4.3		3.5	1.1	
1	58	62	15.5	17.8							

\* Average of six rats

† Average of two rats.

The results of these determinations are summarized in Table I. It may be seen at a glance that the blood plasma phosphate of young growing rats was not increased in chronic fluorine poisoning induced by feeding a ration containing 0.1 per cent sodium fluoride.

In comparing the plasma phosphatase value of the normal control animals with those of the fluorine-fed animals it is interesting to note first, the decrease in plasma phosphatase with age in the control animals, and second, the somewhat lower values in the

fluorine-fed animals of the same age up to 70 days and the somewhat higher values thereafter. The decrease in plasma phosphatase with age in the normal animals parallels the rate of growth and skeletal development. Hammett (14) noted an abrupt decline in bone growth in young rats when from 65 to 75 days of age. In a series of calcium and phosphorus balance experiments in the authors' laboratory (6) a striking decline in the retention of calcium and phosphorus was noted at approximately 70 days of age. In the present investigation, an abrupt decline in plasma phosphatase content may also be noted after the rats have reached the age of 70 days.

The plasma phosphatase of the fluorine-fed animals is lower at first than that of the controls but also decreases with age, although the decrease is more gradual. The drop at 70 days of age is less marked than that of the control animals and higher phosphatase values are thereafter noted in the fluorine-fed animals. Here again, the results parallel our previous findings that animals fed 0.1 per cent sodium fluoride in their ration are stunted in growth and bone development and retain less calcium and phosphorus than do the control animals up to the age of 70 days but the retention of these elements is higher thereafter. This difference is probably due to the slower rate of development and calcification of the stunted fluorine-fed animals which was extended over a longer period of time.

The phosphatase content of the bones of the two groups of animals shows no measurable difference until the age of 70 days, at which time the bone phosphatase values of the fluorine-fed animals are greater, again probably indicative of more active calcification as compared with the controls which have passed the period of most active bone growth and, therefore, show lower bone phosphatase content.

The incisor teeth of the fluorine-fed animals after 2 weeks on a ration containing 0.1 per cent sodium fluoride show no difference in phosphatase content. However, when the animals are continued on this diet, a very positive decrease of the content of the glycerophosphate-splitting enzyme was observed and the incisors of fluorine-fed animals showed a lower content of this enzyme than did the teeth of the control litter mate rats. The rate of eruption of the incisor teeth of rats on these diets has been previously



measured (15) and it was found that the incisors of rats on the fluorine Diet 214 grew at a rate of 16.8 mm. in a 10 week period after weaning as compared with a 31.7 mm. growth of the incisors of the control animals in the same period of time. Growth of the incisors was practically normal during the first 2 weeks but markedly retarded thereafter, with almost cessation of growth after 8 weeks on the fluorine ration. Thus it appears that the phosphatase content of the incisor teeth is dependent upon the activity of their growth and calcification.

The lower phosphatase values of plasma, bones, and teeth of the fluorine-fed animals during the period of most active growth of the normal animals seemed to be best explained secondary to the growth and calcification stunting effect of fluorine instead of any specific effect of fluorine upon the phosphatase enzyme itself. Further corroborating evidence may be seen in the phosphatase values of the plasma, bones, and teeth of rats receiving sufficient fluorine in their ration (0.025 per cent sodium fluoride) to mottle the teeth but not a high enough concentration to interfere with the rate of growth of the animals or give any evidence of abnormality of skeletal development. As may be seen in Table I rats on Diet 216 had phosphatase values practically the same as those of the control non-fluorine-fed rats of the same age, litter, and sex.

*Series II*—In order to test further the effect of fluorine upon the phosphatase enzyme, an acute fluorine poisoning was produced in rats by the subcutaneous injection of a solution of sodium fluoride. Schour and Smith (10) have shown that the single injection of 0.3 cc. of a 2.5 per cent solution of sodium fluoride produced marked gross and histologic changes in the incisor teeth.

Accordingly, male albino rats taken from the stock colony (basal Diet 13) were given subcutaneous injections of 0.3 cc. of 2.5 per cent sodium fluoride solution. The animals were then fasted overnight, at which time (18 hours after injection) the blood was sampled and plasma phosphatase determined in the usual manner. The period of active growth and sex differences was avoided by using only male rats 14 weeks of age or older. As before, comparisons are made between the plasma samples of litter mate rats analyzed at the same time. Most of these analyses were made in the summer months, which may explain the slightly higher values found for the few animals of the same age as compared with those reported in Series I.

The results of this study appear in Table II. While in a few cases the injected animals had a slightly higher plasma phosphatase content than the control animals, in other cases the reverse was true and in most cases no difference could be noted. In no cases were the differences of the striking order reported by Phillips as occurring in fluorosis in dairy cows

The average plasma phosphatase values for nineteen injected rats of different adult ages was 18.9 units as compared with 18.7 units for nineteen of their non-injected litter mate controls. Thus it may be concluded that the concentration in the plasma of the enzyme probably involved in tooth and bone calcification was not

TABLE II  
*Effect of Injection of Sodium Fluoride upon Plasma Phosphatase of Albino Rats*

No of rat pairs	Age <i>wks</i>	Average phosphatase content, mg P liberated per 100 cc plasma	
		Control rats	Injected rats
5	14	17.8	19.7
4	16	18.0	18.8
2	19	16.4	16.6
1	20	29.0	24.0
3	24	13.0	14.4
3	27	18.1	17.0
1	41	19.0	22.3
Average of 19 adult rats		18.7	18.9

increased in acute fluorine poisoning 18 hours after the injection of a solution of sodium fluoride.

#### SUMMARY

A study has been made of the effect of sodium fluoride upon the phosphatase content of the plasma, bone, and incisor teeth of albino rats. Comparisons were drawn between analyses made at the same time on fluorine-fed and non-fluorine-fed rats of the same age, sex, and litter. The plasma phosphatase values of the normal control rats decreased with age, a rather precipitous dip occurring at about 70 days of age, with little change thereafter.

The plasma phosphatase value of the animals whose ration con-

tained 0.1 per cent sodium fluoride also decreased with age, but more slowly. They are lower than those of the control rats until the age of approximately 70 days, after which they are slightly higher. It is believed that the lower values noted are an indication of less active bone growth and delayed maturity rather than a specific fluorine effect.

Animals receiving 0.025 per cent sodium fluoride in their ration, a concentration sufficient to mottle the teeth but not to stunt bone development or produce other signs of fluorosis, show phosphatase values of the same order as the control rats.

The phosphatase content of the incisor teeth of the animals on the high fluorine diet is less than that of the controls. Previous work has shown that their rate of eruption is inhibited by fluorine feeding.

Little difference was seen in bone phosphatase values of the controls and their fluorine-fed litter mates until about 70 days of age, after which time they were slightly higher in the fluorine-fed rats.

The plasma phosphatase values in adult rats, determined approximately 18 hours after the injection of 0.3 cc of a 2.5 per cent sodium fluoride solution, were not significantly different from those of the controls.

It is concluded that fluorine does not exert its characteristic damage to the teeth of rats through its effect upon the enzyme involved in tooth and bone calcification, nor can an increase in plasma phosphatase content be considered a sensitive indication of fluorosis in rats as reported by Phillips (8) for dairy cows.

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# AN IMPROVED APPARATUS FOR THE DETERMINATION OF COLLOID OSMOTIC PRESSURE IN SMALL AMOUNTS OF FLUID

BY REUBENIA DUBACH AND ROBERT M. HILL

*(From the Department of Biochemistry, University of Colorado School of  
Medicine, Denver)*

(Received for publication, August 19, 1935)

The need for a convenient and practical method for the measurement of the colloid osmotic pressure of biological fluids is evident from the number of methods which have recently been described.

Modifications of Sørensen's (1) collodion sack method by Krogh (2), Mayrs (3), and Wells (4) have yielded excellent results in the hands of these and other investigators but require a careful technique in the preparation of the membranes. The ingenious method of Schade (5) (which has been adopted and modified by Kylin and von Pein (6)) has not found general application. Perhaps the most promising method from the standpoint of adaptability to routine laboratory use is the second method described by Krogh and Nakazawa (7) in which a hard rubber osmometer and flat commercial membranes are employed. A micromodification of this method by Hill (8) reduces the amount of fluid required to 0.3 cc. and shortens the time of equilibration.

The advantages of the Krogh-Nakazawa osmometer over others now in use are (1) the convenience of manipulation, (2) the small amount of fluid required, (3) the short time required to establish equilibrium, and (4) the use of inexpensive commercial membranes which require very little expenditure of time in preparation for use. In spite of these advantages, we have had to discard more than one-third of our determinations with this osmometer because equilibrium was never obtained. After studying the possible causes of this difficulty we have come to the conclusion that the fault is in the use of rubber washers to hold the membrane in

place. When pressure is applied in assembling the osmometer there is some flattening and spreading of the washer. This causes a bulging of the center of the membrane and in some cases it loses contact over most of its surface with the filter paper holding the outer fluid. When washers hard enough to prevent this bulging are used, they frequently do not prevent leaking.

With a view to eliminating this source of error we have constructed an osmometer of brass with parts so machined that they fit together tightly without the use of washers. The construction is based on that of the Krogh-Nakazawa apparatus but differs from it in a number of essential details. This osmometer has been in use in our laboratory for over 2 years.

The osmometer is illustrated in Figs. 1 and 2. Fig. 1 shows the separate parts and Fig. 2, a longitudinal section of the assembled apparatus. The perforated disk, *e*, supports a disk of filter paper, *k*, which bears the outer fluid, and above it the collodion membrane, *j*.<sup>1</sup> This disk rests in a depression in the top of the cone, *f*, and is held firmly in place by the collar, *d*. The disk is 0.001 inch thicker than the depression in which it rests. This serves to keep the membrane tight against *d*. The ring, *g*, screws into *d* and forms a rigid support for *f*. The parts, *d*, *e*, *f*, and *g*, are shown assembled in Fig. 2. When in position they form the well, *z*, which holds the plasma. The bottom of this well is the membrane, now held firmly in place; the wall is the upper part of the rim of *d*. The cap, *c*, forms the top of the osmometer and completes the cell containing the plasma. The capillary tube, *a*, which dips into the plasma through the opening in the cap, *c*, is held in place by the box screw, *b*, which screws over the rubber washer, *h*. The washer serves to prevent leaks and also to hold the capillary tube firmly in position.

Fig. 2 shows a longitudinal section of the assembled osmometer with the parts *g*, *f*, *e*, *d*, and *c*, all closely in apposition, and the bottom of the capillary tube, *a*, dipping into the cell, *z*, which holds the plasma. The disk of filter paper and the membrane are shown in the longitudinal section by a single line above the disk, *e*.

<sup>1</sup> *Ultrafeinfilter* (120 minute grade), obtained from Membranfilter-Gesellschaft, m. b. H., Göttingen. Turner (9) has recommended cellophane No 600 as a suitable membrane for use in the Krogh osmometer. In our hands this membrane has given erratic results.

The advantages of this apparatus over the Krogh-Nakazawa osmometer are:

1. The osmometer is made of brass so machined that the parts fit together tightly and do not require washers to prevent leakage around the membrane. This insures a flat membrane throughout

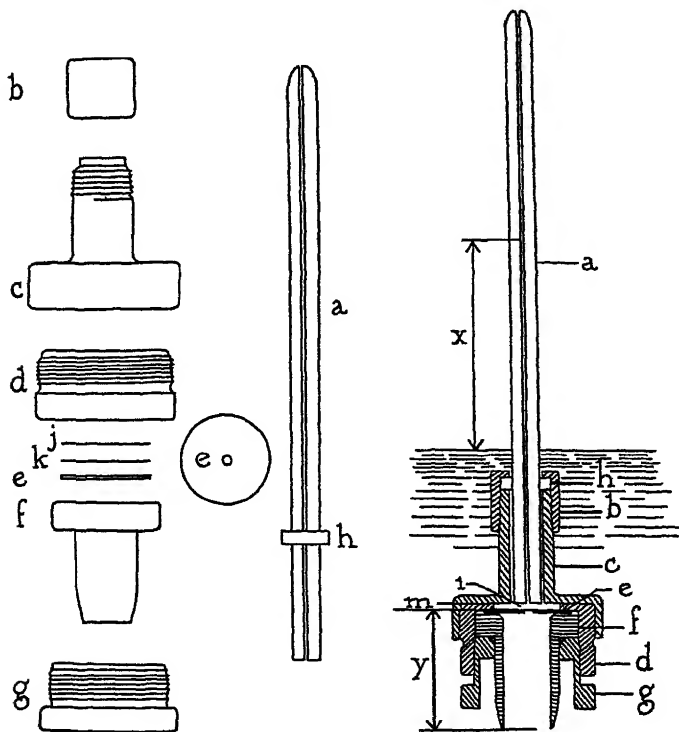


FIG. 1

FIG. 2

FIG. 1. Separate parts of osmometer

FIG. 2. Longitudinal section of the assembled apparatus.

the determination without any of the bulging that is almost unavoidable in an apparatus which holds the membrane in place with a rubber washer.

2. The membrane is still in view after it has been put in place, so that if any accident has occurred to it while assembling the



parts the defect can be seen; also any outside fluid which has leaked around the edge of the membrane can be wiped off.

3 The plasma is introduced into the cell before the cap, *c*, is applied. Hence any bubbles that may form around the edges can be seen and removed.

4. When it is desired after a determination to test the outer fluid for leakage of protein through the membrane, the cap, *c*, can be removed and the inner solution completely washed out before the rest of the apparatus is taken apart. Thus there is no chance of contamination of the outer fluid with the inner fluid after the determination is completed. The advantages of this will be apparent to those who have had experience with the Krogh-Nakazawa osmometer.

The arrangement described by Turner (9) for making several determinations at the same time is employed. The osmometers are hung from the side arms of a horizontal brass tube sealed at one end and connected at the other with a water manometer. The following modifications have been made. (1) The capillary tubes are left square at the top. (2) Heavy rubber pressure tubing is used to connect the glass tubes with the brass tree. This is necessary in order to support the weight of the brass osmometers. (3) Glass stop-cocks are inserted between the brass tube and the individual capillary tubes instead of using pinch-clamps on the rubber tubes. These have the advantage that turning the plug in the glass stop-cock does not alter the volume of the system.

The lower parts of the osmometers dip into a glass jar containing 0.9 per cent sodium chloride solution to prevent the evaporation of the outer solution from the filter paper

The determination is conducted as follows. A disk of filter paper moistened with 0.9 per cent sodium chloride solution is placed over the brass osmometer disk, *e*, which is in position on top of the cone, *f*. A disk of membrane is then applied, care being taken that no bubbles are caught between the filter paper and the membrane. The membrane is held in position by placing the collar, *d*, over the cone, *f*. These parts are then inverted and the part, *g*, is screwed into *d*. During this operation *g* is the only moving part. A shearing motion between *f* and *d*, which might injure the membrane, is thus avoided. After the parts *d*, *e*, *f*, and *g* have been assembled, the membrane is still visible and can be examined

for flaws before the plasma is put in. Excess of moisture is removed from the surface of the membrane by blotting it with a small piece of clean filter paper. The plasma is added immediately from a fine tipped pipette until the level of the liquid forms a rounded meniscus above the rim of the chamber. The cap, *c*, is then screwed into place over *d*. The surfaces of *c* and *d* where they touch at *m* (cf. Fig. 2) are so machined that they fit without leaks. Before inserting the capillary tube, *a*, a wire which has been dipped in caprylic alcohol is touched to the surface of the plasma to break any air bubbles which may have formed in screwing on the cap. The capillary tube, *a*, bearing the rubber washer, *h*, at the proper distance from the end, is then carefully inserted through the hole in the top of *c* and the box nut, *b*, is screwed into position over the washer. If the osmometer has been properly filled, the tightening of the screw upon the rubber washer causes the plasma to rise in the tube to a height of 8 to 10 cm. The washer, *h*, must have a thin coat of vaseline so that it will slide on the metal parts *b* and *c*; otherwise, in assembling, the shearing force will tear the rubber and cause leaks.

When thus assembled the osmometer is connected with the pressure system previously described. The stop-cock is kept open while the tube is being inserted in order to avoid too sudden an increase in pressure. As soon as the osmometer is in position the leveling bulb of the water manometer is raised in order to put upon it approximately the pressure expected. The stop-cock is then closed until a reading is to be taken.

The time required for the establishment of equilibrium varies generally from 3 to 6 hours. Readings are begun at the end of 3 hours and continued at half hourly or hourly intervals until two successive readings check within 10 mm. The method of making a reading is the same as with the Krogh apparatus. The leveling bulb of the water manometer is raised to a level approximating the pressure expected; then the stop-cock is opened and the leveling bulb is adjusted until the column of plasma in the capillary tube becomes stationary. The meniscus is observed for several minutes through a low power reading microscope with a cross-hair in the ocular. When it has attained a constant level, the following readings are made: (1) the height of the counterbalancing column of water in the manometer, and (2) the height of the fluid

in the capillary tube above the level of the saline solution. These two readings are added together and two negative corrections deducted: (1) the capillary effect of the osmometer tube, and (2) the depth of the cone,  $f$ .

Total pressure =  $A + B - p$ , in which  $A$  = the pressure of water on the leveling stand,  $B = x - y$ , and  $p$  = the capillarity in the osmometer tube. As shown in Fig. 2,  $x$  = the height of the plasma above the level of the saline solution and  $y$  = the depth of the cone,  $f$ .

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## A MODIFIED REHBERG BURETTE FOR USE WITH TITRATING SOLUTIONS WHICH REACT WITH MERCURY

BY BERNARD B LONGWELL AND ROBERT M. HILL

(From the Department of Biochemistry, University of Colorado School of Medicine, Denver)

(Received for publication, August 19, 1935)

Rehberg<sup>1</sup> has described a simple, highly accurate microburette which is very useful for certain types of titration. Its use is limited, however, to titration with solutions which do not react with mercury. In order to overcome this difficulty we have modified the burette so that mercury does not come in contact with the titration fluid.

The parts of the apparatus are shown in detail in Fig. 1. With the exception of parts *a* and *b* and the rubber diaphragm held between them, the apparatus is essentially the same as that of Rehberg. The 2 cc. Record syringe, *m*, which is used, is selected for the perfect fit of the plunger. The stem *s* is threaded and moves in the block *o*, which is fastened to the metal cap of the syringe barrel. The block should be at least  $\frac{3}{8}$  inch thick. A short piece of heavy rubber tubing, *p*, slipped over the handle of the adjusting screw is an aid to delicate manipulation. When in use the plunger is coated with a heavy stop-cock grease. The cement with which the tip, *t*, is usually fastened to the barrel of the syringe, as it comes from the supply house, will not hold mercury. For this reason it is removed, the cement scraped away, and the tip replaced, cellulose paste<sup>2</sup> being used to secure it to the barrel. Consecutive rings (not a thread) are cut in the tip, *t*, to secure it in the fine bore pressure tubing, *l*, which serves to hold the syringe to the burette, *c*. To make this connection

<sup>1</sup> Rehberg, P. B., *Biochem J*, 19, 270 (1925).

<sup>2</sup> Two sheets of cellulose acetate photographic film, 4 × 5 inches, are washed free of gelatin, dried, and dissolved in 100 cc. of a mixture of 83 parts of dry acetone and 17 parts of absolute alcohol.

firm, the tip, *t*, the pressure tube, *l*<sub>1</sub>, and the upper half inch of the syringe barrel are covered with several coats of cellulose paste. The connections, *l*<sub>2</sub> and *l*<sub>3</sub>, are of the same heavy walled tubing as *l*<sub>1</sub>, but need not be coated with cellulose paste. For the burette, *c*, we use carefully calibrated, graduated pipettes of 0.1, 0.2, or 1.0 cc. capacity. The glass parts, *a* and *b*, are exactly alike, and

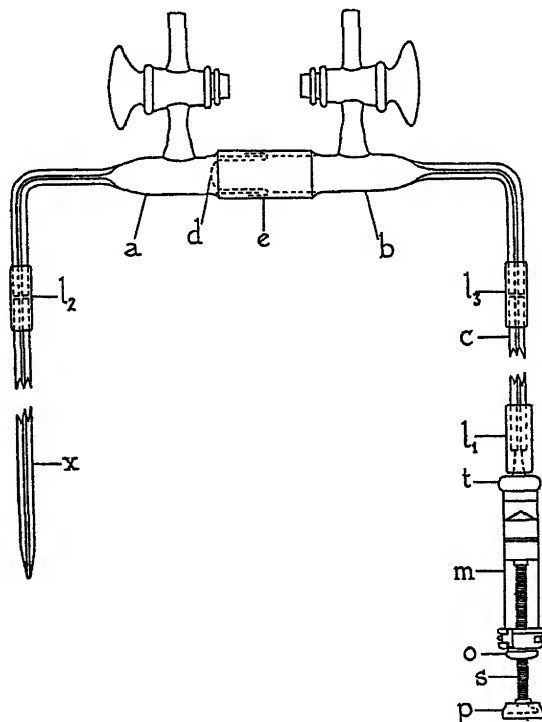


FIG. 1

each should have a capacity of 3 or 4 cc. The rubber diaphragm, *d*, is a finger cut from a rubber glove and inserted into part *a*. The cut end of the finger is turned back over the outside of *a*, where it should fit tightly. The piece of rubber tubing, *e*, holds the parts, *a* and *b*, together and keeps the diaphragm, *d*, securely in place. To prevent expansion of the tubing, *e*, it may be fitted with a metal collar, or it may be wrapped with a coil of copper wire and coated with cellulose paste.

The first step in filling the burette is the introduction of 2 cc. of mercury into the syringe. It is sometimes more convenient to do this before the syringe is attached to the burette, or it may be introduced through the stop-cock in *b* by lowering the plunger of the syringe. If the latter method is used, the apparatus must be tipped to avoid trapping mercury in *b*. After the mercury is introduced, *b* and *c* are completely filled with water through the stop-cock in *b*, by raising and lowering the plunger of the syringe. When this is accomplished the stop-cock in *b* is closed. Part *a* and the tip, *x*, must be completely filled. To accomplish this, the tip, *x*, is dipped below the surface of some titrating fluid in a test-tube, and suction is applied at the stop-cock in *a*. When *a* is filled, its stop-cock is closed. Care must be taken to remove all bubbles of air. Finally the stop-cock in *b* is opened, and the plunger of the syringe is raised until the mercury stands above the calibration on the burette; this stop-cock is then closed. The burette is now ready to be filled for use. The titrating fluid is introduced through the tip, *x*, and the mercury brought to the zero point on the burette by manipulating the syringe plunger.

The use of the burette is as simple as is that of Rehberg. We have not determined how long the diaphragms will remain in good condition, but one in use with thiosulfate solution has not needed attention for nearly a year.

For a complete discussion of the use of microburettes and the theory of microtitrations, the reader is referred to the original paper of Rehberg.<sup>1</sup>

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## SPECTROSCOPIC INVESTIGATIONS OF AMINO ACIDS AND AMINO ACID DERIVATIVES

### I. ULTRA-VIOLET ABSORPTION SPECTRA OF *L*-TYROSINE, *dl*-PHENYLALANINE, AND *L*-TRYPTOPHANE

BY KATHERINE FERAUD, MAX S DUNN, AND JOSEPH KAPLAN  
(From the Chemical and Physical Laboratories, University of California at  
Los Angeles, Los Angeles)

(Received for publication, August 22, 1935)

The absorption spectra of the amino acids in the ultra-violet region are of interest, particularly because of their basic importance in the determination of protein composition by spectroscopic methods. The experiments described in the present paper were undertaken by the authors because the absorption data reported by earlier workers (1-15) are incomplete and not wholly concordant. Quantitative absorption characteristics of the amino acids in the ultra-violet and other wave-length regions will be reported in subsequent papers.

#### EXPERIMENTAL

The phenol, benzene, *L*-hydroxyproline, *L*-proline, *L*-tryptophane, and *L*-histidine dihydrochloride used in this work were the highest grade obtainable from commercial concerns. Indole, *L*-tyrosine, *dl*-alanine, *dl*-leucine, and *dl*-phenylalanine were highly purified samples prepared in this laboratory.

The apparatus consisted of a small quartz Gaertner, equivalent to the Hilger spectrograph E-31, and a hydrogen discharge tube of transparent fused quartz. Although the latter contained a trace of carbon monoxide, fortunately the radiations from the impurity were in a wave-length region removed from that of amino acid absorption. The amino acids were dissolved in distilled water and placed in a fused quartz cylinder, 2.5 by 24 inches. The solutions were exposed to the discharge from the hydrogen tube for an average time of about 30 minutes. In the case of extreme dilutions 3



hour exposure periods were employed. Exposures were made at increasing dilutions until the maximum number of absorption bands was found. The wave-lengths of the absorption bands were measured by comparison with the lines in the mercury arc spectrum. Eastman No. 33 plates were used to photograph the absorption spectra.

TABLE I

*Absorption Bands of l-Tyrosine, Phenol, dl-Phenylalanine, Benzene, l-Tryptophane, and Indole in Ultra-Violet Region*

<i>dl</i> -Phenylalanine	Benzene	<i>l</i> -Tyrosine	Phenol	<i>l</i> -Tryptophane	Indole
$\lambda$	$\lambda$	$\lambda$	$\lambda$	$\lambda$	$\lambda$
2675	2685	2820	2760	2894	2873
2643	2645	2760	2690	2804	2790
2576	2605	2680	2625		2710
2525	2545	2605			
2462	2480	2540			
2410	2420	2470			
2350	2380	2415			
	2330				

Several indistinct bands at shorter wave-lengths than those shown in the table were observed in each case for benzene and phenol. Baly and Collie (16) observed seven absorption bands for benzene of the wave-lengths 2684, 2655, 2611, 2554, 2484, 2432, and 2381 Å. Eight absorption bands of the wave-lengths 2674, 2594, 2535, 2477, 2426, 2372, 2329, and 2290 Å. were reported by Henri (17). When phenol was dissolved in pentane, Klingstedt (18) found three intense absorption bands at the wave-lengths 2772, 2705, and 2643 Å; one or two feeble bands at 2550 Å, and two broad bands at 2150 and 2040 Å. When phenol was dissolved in water, only one broad band could be observed. Absorption bands at 2869, 2806, 2788, 2762, 2650, and 2170 Å. were reported by Friedli (19) for indole. Bands at almost identical wave-lengths were observed by Menczel (20).

### Results

It was found that *dl*-alanine, *dl*-leucine, *l*-histidine dihydrochloride, *l*-proline, and *l*-hydroxyproline gave general absorption in the ultra-violet region, while benzene, phenol, indole, *l*-tyrosine, *dl*-phenylalanine, and *l*-tryptophane showed selective absorption. These results are in accord with the findings of previous workers and with the commonly accepted view that the aromatic hydro-

carbons are the only compounds which show banded structure in their spectra

The spectra of the compounds which exhibited selective absorption are shown in Figs 1 to 3. The wave-lengths of the absorp-



FIG. 1



FIG. 2

FIG. 1 The upper spectrum represents phenol, the lower, *L*-tyrosine. The light portions of the photographs indicate absorption bands. The photographs of the two spectra are fixed in positions which correspond to the same wave-length scale. The concentration of the amino acid solution was found to be important. At a concentration of 0.13 gm per 100 cc of distilled water only a broad region of absorption appeared. On further dilution to a concentration of 0.026 gm per 100 cc the maximum number of bands was detected.

FIG. 2 The upper spectrum represents benzene, the lower, *DL*-phenylalanine. The light portions of the photographs indicate absorption bands. The photographs of the two spectra are fixed in positions which correspond to the same wave-length scale. The maximum number of bands was found at a concentration of 0.075 gm per 100 cc for *DL*-phenylalanine and 0.036 gm per 100 cc for benzene.



FIG. 3 The upper spectrum represents indole, the lower, *L*-tryptophane. The light portions of the photographs indicate absorption bands. The photographs of the two spectra are fixed in positions which correspond to the same wave-length scale. The maximum number of bands was found at a concentration of 0.0038 gm per 100 cc for *L*-tryptophane and 0.0034 gm per 100 cc for indole.

tion bands in Angstrom units are given in Table I. The bands which are not distinguishable in the reproductions of the photographs were clearly visible on the original plates.

As indicated in Fig 4, three of the bands for *l*-tyrosine, five for *dl*-phenylalanine, and two for *l*-tryptophane, reported by other workers, agree closely with bands found in the present investigation. However, two new bands for *dl*-phenylalanine and four for *l*-tyrosine have been discovered in the spectra of these amino acids

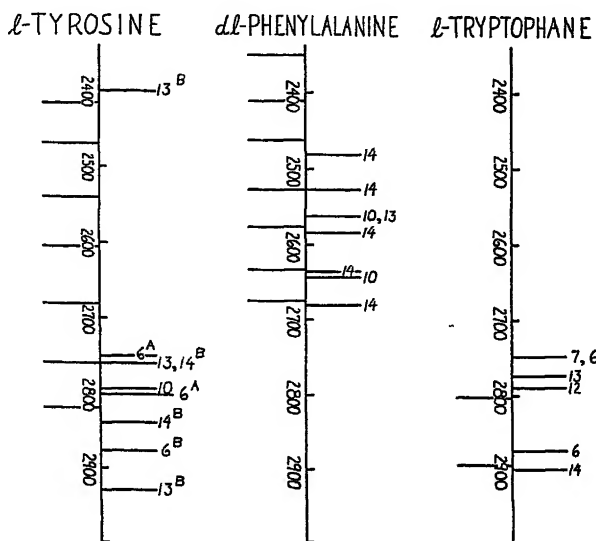


FIG 4 Absorption bands of *l*-tyrosine, *dl*-phenylalanine, and *l*-tryptophane in the ultra-violet region. The horizontal bands to the left of the vertical lines indicate absorption bands found by the authors, while the horizontal bands to the right are what seem to be the most reliable values in the literature. The numbers to the left of the vertical lines are wave-lengths in Ångstrom units, while the numbers to the right of the horizontal lines refer to bibliographic citations. The letters *A* and *B* signify that the bands shown for *l*-tyrosine were obtained in acid (*A*) or basic (*B*) solution.

Ross (14) made the prediction that "phenylalanine would have several sharp bands, while tyrosine and tryptophane would have only one or two diffuse bands." It appeared to us more probable that the spectra of tyrosine and phenylalanine would be nearly the same, since their absorbing nuclei differ only by a hydroxyl group. On the other hand, it would be logical to look for a definitely different spectrum in the case of tryptophane, since its

absorbing nucleus is a structure formed from the fusion of benzene and pyrrole. The authors' experimental data appear to lend support to these hypotheses

By determining the parts by weight of each amino acid required for characteristic absorption in a given amount of solution in a standard cell, Ross reported 1.0, 0.50, and 0.05 as the ratio of the absorbing strengths of tryptophane, tyrosine, and phenylalanine. When the parts by weight required to produce the maximum number of bands were used in a similar calculation with the authors' data, the same relative order was found

It is difficult to harmonize the present experiments with the statement, made by Ross, that phenylalanine, of all the amino acids, has an absorption spectrum peculiar to itself, which may be used for its identification. Since it appears that the absorption bands of tyrosine and phenylalanine lie in nearly the same wavelength region, it seems evident that one of these amino acids must be absent if the determination of the other by spectroscopic methods is to be reliable. On the other hand difficulties from interference by other amino acids would be less significant in the spectroscopic determination of tryptophane.

Numerous attempts (1, 2, 6, 9, 12, 13, 15, 21, 22) have been made to explain the absorption spectra of proteins in terms of the individual aromatic amino acids. While investigations with gelatin, a protein known to contain phenylalanine but only a small amount of tyrosine and no tryptophane, might conceivably yield reliable information, it would be less probable that spectroscopic measurements of other, more complete proteins would give absorption bands which could be attributed with certainty to any particular amino acid.

From the data recorded in Fig. 4 it may be seen that the absorption bands of *l*-tyrosine, *dl*-phenylalanine, and *l*-tryptophane closely resemble those of phenol, benzene, and indole respectively. The principal difference seems to be a shifting of the bands to longer wave-lengths. On the whole it may be safely concluded that the aromatic nuclei of these three amino acids are mainly responsible for their selective absorption in the ultra-violet region and that their aliphatic side chains, which exhibit only general absorption, serve merely to produce alterations in the positions of the bands.

## SUMMARY

The ultra-violet absorption spectra of *l*-tyrosine, *dl*-phenylalanine, *l*-tryptophane, benzene, phenol, and indole have been determined. It has been shown that the spectra of the three amino acids resemble those of their corresponding aromatic nuclei. Because of the overlapping spectra of *l*-tyrosine and *dl*-phenylalanine the certain determination of either amino acid in a protein or a mixture of amino acids would seem to be feasible only in the absence of the other form.

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## THE CHEMISTRY OF HUMAN EPIDERMIS

### II. THE ISOELECTRIC POINTS OF THE STRATUM CORNEUM, HAIR, AND NAILS AS DETERMINED BY ELECTROPHORESIS

BY VERNON A. WILKERSON

*(From the Department of Biochemistry, Howard University Medical School,  
Washington)*

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In a previous publication we have shown that the outer layer of human skin is a distinct chemical entity and corresponds to the definition of a keratin as given by Block and Vickery (1). It is, therefore, amphoteric in nature, capable of ionizing both as an acid and as a base, and has a definite isoelectric point.

It has been well established that these outer cells are normally dead, extremely desiccated, and partially keratinized, and that their peculiar properties are responsible for the many phenomena that are of importance in connection with the local application of drugs. Clinically it is a matter of common knowledge that certain substances in solution are readily absorbed through the intact skin, while others are absorbed with difficulty or not at all. Certain ions under the influence of an electrical current permeate the epidermis at varying rates; still others cannot be made to pass the superficial layer, no matter what the conditions are.

The pH of the skin has been determined by Schade and Marchionini (2), Sharlit and Scheer (3), Rastelli (4), and other investigators. The lack of uniformity in results leads to the obvious conclusion that in the living material this value is dependent chiefly upon the amount of perspiration, the oil in the skin, and the condition of the underlying tissues. It would, however, be convenient and quite fundamental to be able to refer such values as are obtained to the actual isoelectric point of the outer epithelium. From the above considerations it was thought that the determination of the isoelectric point would be of practical as well as theoretical significance.

## EXPERIMENTAL

There are a number of methods available for the determination of the isoelectric point of proteins. All depend upon the observation that at this point the osmotic pressure, viscosity, conductivity, swelling, precipitability by alcohol, acid- and base-binding power, and migration in an electrical field are at a minimum. The insolubility of the human epithelium in ordinary solvents and the ease with which microscopic suspensions are prepared readily suggest the possibility of studying the migration of the particle in an electrical field. This is the most direct method and is termed microelectrophoresis.

The material for analysis consisted of the layer of epidermis known as the stratum corneum. Samples were collected from scrapings of normal skin, callouses on the hands of laborers, and exfoliative disturbances from hospital patients. All samples were washed with water, extracted with acetone, alcohol, and ether, dried at 105°, and pulverized in a ball mill.

The powdered skin was suspended in a series of acid potassium phthalate buffers (5) whose pH ranged from 1.39 to 6.13. The pH of each of the solutions was determined immediately before being used by employing the quinhydrone electrode. Measurements of the electrophoretic velocity were made with a modified Northrop-Kunitz apparatus in which a flat microelectrophoresis cell whose width was very large as compared to its thickness was utilized. The theory concerning such a cell has been completely discussed in the recent monograph by Abramson (6).

Three radio batteries giving approximately 135 volts were connected at each end of the cell to non-polarizable electrodes of copper in saturated copper sulfate solution. Dental plaster of Paris plugs were found to be most useful in impeding diffusion.

A 20 × Zeiss ocular and a Bausch and Lomb 8 mm 21 × objective combined working distance with sufficient magnification. The eye-piece, of course, contained a micrometer scale which had in turn been accurately graduated against a Zeiss stage micrometer.

Since velocities are given in  $\mu$  per second per volt per cm, the potential drop per cm or field strength must be determined. As the cell is uniformly rectangular and the field is practically homo-

geneous throughout, the potential gradient  $X$  can be directly obtained from Ohm's law,  $X = (IR)/Q$ , where  $I$  measures current;  $R$ , resistivity of the suspension; and  $Q$ , cross-section of the cell.

The current  $I$  was measured by a microammeter which was introduced in the circuit and the resistivity  $R$  of the suspension was measured at the beginning of each experiment by means of the Wheatstone bridge. This method, preferred by Abramson (7), avoids the errors due to accidental resistances when the potential gradient is taken simply as the applied E.M.F. divided by the length of the cell. The current was reversed by means of a Pohl mercury commutator.

Owing to the charge which the glass assumes against the water and the subsequent endosmotic steaming when the circuit is closed, it was necessary, in order to eliminate this source of error, to measure the movement of the suspended particles in successive layers from the top to the bottom. The movement of liquids and suspended particles in flat electrophoresis cells has been investigated by many workers including Ellis (8) and Smoluchowski (9). The latter, as a result of theoretical considerations based upon sound thermodynamic principles, has formulated that there are stationary levels at approximately 0.21 and 0.79 of the total depth of the cell. By the use of the quartz crystals (10) our cell was found to conform to the theory of Smoluchowski.

All the work was done at 25°. Preliminary experiments showed that the electrophoretic activity of the samples of skin was the same regardless of the source.

#### DISCUSSION

The data of the observed velocities in  $\mu$  per second per volt per cm. at a definite pH are plotted in Fig 1, which shows the shape of the pH-mobility curve. Each point represents the average of ten observations. The value assigned to the isoelectric point of the stratum corneum is 3.70.

Rein (11), investigating the migration of anions and cations in the intact human skin, observed that between pH 3 and 4 a change of the sign of the charge occurred. The isoelectric point of 3.7 obtained by the pH-mobility curve is in good agreement with this region of reversal of sign.

Similarly, the observed velocities were determined for human



hair and human finger nails. The values assigned to the isoelectric points were respectively, 3.67 and 3.78 (Figs. 2 and 3)

It is observed that the isoelectric points of human skin, hair, and nails are very nearly the same. However, two or more amphoteric substances may have the same isoelectric point on the pH scale and yet be totally different in composition. A comparison of such points is therefore distinctly limited and is only of true value when used to show similarities or differences between substances within a natural group. Skin, hair, and nails have been so considered, because by their physical properties they were originally classified as keratins. Again it has been shown quite recently that they have the same basic anlage. The ratio of histi-

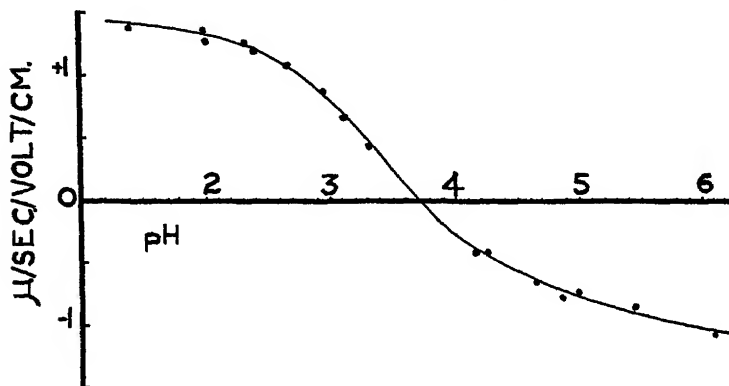


FIG. 1. pH-mobility curve and assigned isoelectric point (3.70) of skin

dine, lysine, and arginine (1) in each has been found to be approximately 1:5.15, and so they conform chemically to the definition of keratins. Finally, the epidermis, hair, and nails are all derived embryologically from the ectoderm, while histologically the nails and hair are usually considered as specialized and highly keratinized appendages of the epidermis.

It has been shown by different workers, but especially by Cohn (12), that the acid- and base-binding capacity of proteins is connected with their amino acid content and consequently the numerical value of the isoelectric point is a function of the ratio between the strengths of the acidic and basic groups. As a matter of fact, the position of the isoelectric point in naturally related substances

may indicate the relative number of such groups. It is assumed that the basic groups are chiefly those of the  $\epsilon$ -amino group of lysine, the guanidine group of arginine, and the imidazole group of histidine, while the chief acidic groups are the free carboxyl groups of the dicarboxylic acids, and the hydroxy group of tyrosine.

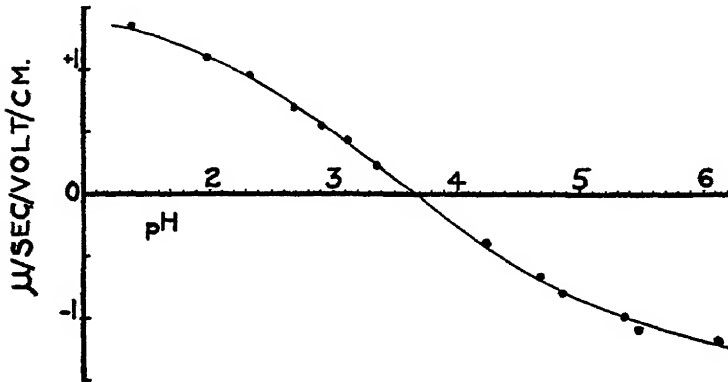


FIG. 2 pH-mobility curve and assigned isoelectric point (3.67) of hair

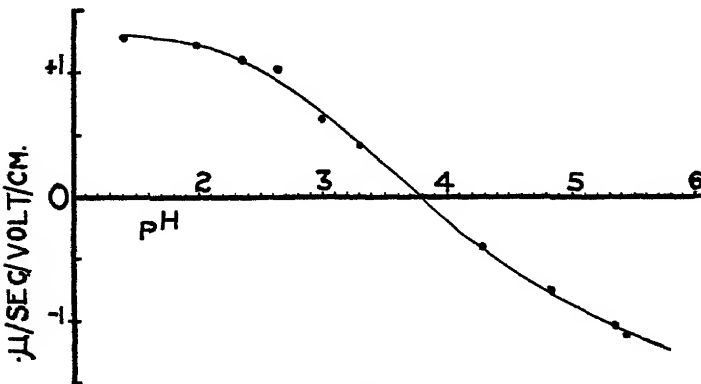


FIG. 3. pH-mobility curve and assigned isoelectric point (3.78) of nails

From the similarity of the isoelectric points of the skin, hair, and nails and from the previous observation that the basic amino acids, lysine, arginine, and histidine, were present in the same molecular ratio it might be predicted that the amino acids responsible for the acidic groups of these three closely related tissues are also

present in a somewhat definite molecular ratio. It must be added, however, that while the isoelectric points are very nearly the same, the slope of the curve in each instance is different. This indicates the different manner in which the ions present in the buffer affect the velocity of the suspended particles.

In reviewing the work on the pH of the skin it is noted that most of the values reported are on the alkaline side of the isoelectric point. It is significant, however, that in the work of Schade and Marchionini (2), the pH of the human skin was found to vary from 1.78 to 5.17, with an average pH of 3.78. This is remarkably close to the isoelectric point of the superficial layer.

In the local application of colloidal suspensions of a medicinal or cosmetic nature to the thoroughly cleansed epidermis, the adherence depends greatly upon the charge on the suspended particle and the charge that the skin assumes in contact with the solution. This latter would depend upon the isoelectric point of the skin and the pH of the colloidal suspension. Should the pH of the solution fall on the acid side of the isoelectric point, the outer layer of the epidermis would be expected to assume a positive charge and positively charged particles in suspension would be repelled and easily removed, while negatively charged particles would be closely adhered to the surface and consequently removed with difficulty. On the other hand, should the pH of the medium be on the alkaline side of the isoelectric point, the skin would assume a negative charge and the opposite behavior with respect to charged particles would be expected.

#### SUMMARY

The skin, hair, and nails were suspended in a series of buffers and their electrophoretic mobilities determined in a modified Northrop-Kunitz microelectrophoresis apparatus. The curves of mobility in  $\mu$  per second per volt per cm plotted against pH proved to be fairly constant. From this the following isoelectric points were assigned: skin 3.70, hair 3.67, nails 3.78.

The suggestion was made that, since the isoelectric points were practically the same and the basic amino acids were present in approximately the same molecular ratios, possibly the amino acids responsible for the acidic groups were also present in a definite molecular ratio in these three chemically, physically, and embry-

ologically related structures. It has been further suggested that the adherence of colloidal particles in suspension to the skin would depend upon the pH of the dispersion medium, the charge that the skin assumed in contact with the colloidal suspension, and the charge on the suspended particle.

I wish to express my appreciation to Dr. Numa P. G. Adams, Dean of the School of Medicine, Howard University, whose continued work in administration and organization has made this research possible.

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## STUDIES ON MAGNESIUM DEFICIENCY IN ANIMALS

### VII. THE EFFECTS OF MAGNESIUM DEPRIVATION, WITH A SUPERIMPOSED CALCIUM DEFICIENCY, ON THE ANI- MAL BODY, AS REVEALED BY SYMPTOMATOLOGY AND BLOOD CHANGES\*

By HARRY G DAY, H D KRUSE, AND E V MCCOLLUM

(From the Biochemical Laboratory, School of Hygiene and Public Health, the  
Johns Hopkins University, Baltimore)

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In previous papers dealing with the effect of magnesium deprivation on calcium metabolism (1) and on bone composition (2), the results led to a renewed consideration of the evidence supporting the view of a physiological antagonism between calcium and magnesium. It was Loeb who, having shown that magnesium ions inhibit rhythmical muscular contractions (3), laid down the assumption that irritability in tissues depended upon the presence of various ions, among them magnesium, in definite proportions (4). Working with plants, Loew (5) had already found that the harmfulness of magnesium compounds to algæ could be neutralized by the addition of calcium compounds. With this background, the theory of antagonism gradually evolved, at times seemingly without design or recognition, from studies on the lower animal forms. For instance, from data presented by Moore (6) it is evident that the toxic effect of magnesium chloride on trout was nullified by the addition of calcium chloride. Shortly thereafter Loeb (7), in reporting that the effect of magnesium on the jellyfish (*Polyorchis*) could be inhibited by calcium, and *vice versa*, definitely asserted the existence of an antagonism between the two ions. The verification of this hypothesis culminated in the demonstration by Meltzer and Auer (8) that the inhibitory effects of magnesium

\* Presented by Harry G Day to the faculty of the School of Hygiene and Public Health, the Johns Hopkins University, in partial fulfilment of the requirements for the degree of Doctor of Science in Hygiene

compounds on rabbits, manifested by general anesthesia and paralysis, could be overcome by injection of a comparatively small quantity of a calcium salt.

During ensuing years, results derived largely from metabolic and bone studies have strengthened the hypothesis, and have broadened its scope to include the concept of a mutual interaction. It has been observed that injection of magnesium salts increases the excretion of calcium salts (9-11), likewise the ingestion of calcium compounds augments the output of magnesium salts (12), although in ingestion experiments the effect is mitigated if a liberal supply of phosphate is simultaneously administered (13, 14). It has been asserted that this loss of calcium from the body, as a result of high magnesium intake, is reflected in inferior calcification of the bones (15-17), but, in the opinion of others (18, 19), the attendant gastrointestinal disturbances have interfered with an unqualified establishment of this point. On the whole, the evidence from all sources, when critically examined, is at one in upholding the validity of the hypothesis.

If the effects of a preponderance of one or the other element have constituted the principal support of the thesis which posits an antagonism, the effects of a reduction in the available amount of one or the other element have not been neglected. In magnesium deficiency there was noted a marked retention of calcium in the animal body, which persisted over a period of some weeks until the onset of nutritive failure, with the consequent loss of calcium (1). Since the animals usually succumbed to convulsions at a comparatively early period, the effect of calcium retention during this time was reflected in hypercalcification of their bones (2). It thus appears that the purported antagonism between calcium and magnesium holds in those instances in which the intake of one is markedly diminished.

Hitherto in all studies bearing upon the principle of an antagonism between calcium and magnesium, the one element has been supplied in usual amounts while the other has been provided on an increased or reduced scale. We have no information concerning the physiological effects of a diminished supply of both elements. Since anesthesia induced by magnesium can be dispelled immediately by administration of calcium, it is a question how the syndrome

of magnesium deficiency would be affected by a concomitant calcium deficiency. The symptomatologies and associated blood pictures of both magnesium deficiency (20-23) and calcium deficiency (24, 25) are known, so that any change in one under the influence of simultaneous deprivation of the other should be readily recognized. Accordingly, these considerations prompted the production of an experimental condition in which a deficiency of calcium was superimposed upon a magnesium deficiency, with the thought of ascertaining whether one syndrome would predominate, whether one would nullify the manifestations of the other, or whether absence of both elements would intensify the syndrome characteristic of each. Specifically, the questions were raised whether tetany would prevail, and whether the course of nutritive failure manifested in magnesium deficiency would be modified by simultaneous lack of calcium.

#### *Technique and Methods*

In all essential details we adhered to the plan which has been previously described for the selection of the animals and for the preparation of the diets (22)

The composition of the salt mixture and of the diets is given.

#### *Salt Mixture*

KCl	1 0
NaHCO <sub>3</sub>	0 7
Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ·24H <sub>2</sub> O	0 508
KH <sub>2</sub> PO <sub>4</sub>	1 7
NaCl	0 5
Total	4 408

#### *Diet Deficient in Both Calcium and Magnesium*

Casein	20 0
Starch	57 6
Salts	4.4
Butter fat	8 0

50 per cent alcoholic yeast extract, 100 cc in 100 gm. of ration, viosterol, 15 drops per kilo of ration

This ration contains approximately 0.002 per cent of calcium and 0.0002 per cent of magnesium



*Control Diet*

Casein . . . . .	20 0
Starch. . . . .	55 56
Salts . . . . .	4 4
Butter fat . . . . .	8 0
CaCO <sub>3</sub> . . . . .	1 5
MgSO <sub>4</sub> 7H <sub>2</sub> O . . . . .	0 545

50 per cent alcoholic yeast extract, 100 cc in 100 gm of ration; viosterol, 15 drops per kilo of ration

This ration contains approximately 0.61 per cent of calcium and approximately 0.054 per cent of magnesium.

The only deviation from the previous program of blood analysis (21) was the inclusion of one additional determination, plasma pH, which was made by the method of Laug (26).

*Results*

*Symptomatology*—As the first indication of abnormality, young dogs deprived of both calcium and magnesium showed an extraordinary alteration in the appearance of their extremities. After 3 to 5 weeks, depending upon the initial age and growth tendencies of the animals, the extremities from the region of the proximal end of the metatarsals downward increased in size and the phalanges began to spread apart. These changes progressed as the survival period was prolonged, until finally the fore extremities were bent sharply at the wrist joint into a right angle; this deformity reduced the height of the fore half of the animal and shifted forward the posture.

Concomitant with the change in the extremities, a gastrointestinal disturbance, which was characterized by periods of diarrhea followed by constipation, set in. As the survival period extended, the failure of intestinal elimination was often prolonged as much as 10 to 15 days. When excretion finally occurred, the feces were formed and black or, as was sometimes the case, they consisted of copious discharges of dark, watery material containing some mucus and often flecks of blood. Autopsy revealed atonic, edematous, inflamed intestines with small diffuse hemorrhages oozing blood from the mucosal surfaces. Throughout the entire period of the deficiency no irregularity appeared in the output or coloration of the urine such as was noted in magnesium deficiency (22).

The food consumption suffered a decline after 4 to 6 weeks, and

continued slowly to diminish as the deficiency progressed (Table I). Although the control animals consumed 1000 to 2000 gm. of ration a week from the 8th to the 12th week, the experimental animals during this time ate only 350 to 600 gm. a week. The muscles of the latter became atrophic, body fat disappeared, and the hair became rough and shaggy; but there was no loss of hair as is characteristic of magnesium deficiency (22).

Edema was evident in the ears after 6 to 10 weeks. Following its sudden appearance the swelling was sometimes so pronounced after a few days that the ears became pendulous, and it persisted throughout the remainder of the survival period with variations in intensity. At times it almost disappeared, only to reappear with marked severity.

Simultaneously with the appearance of edema came a pronounced protrusion of the eyeballs, which once established never abated. Lacrimation occurred only in tetanic attacks. The exophthalmos was a quite striking feature of the calcium-magnesium syndrome and was entirely absent in the simple magnesium deficiency.

After the animals had been on the deficient diet from 3 to 6 weeks, gross changes in the bones appeared. The ribs became so soft that the thorax contracted in its transverse diameter and elongated in its anteroposterior diameter, a circumstance which gave undue prominence to the sternum. Accompanying the softening and consequent deformation of the ribs, slight bowing of the fore legs was seen. Evidence of marked bone resorption was demonstrable by radiograms and autopsy. Sections of the bones revealed much bloody material in the marrow cavity and greatly rarefied shafts. Finally, the bones and joints were so severely affected that they constituted a mass of softened tissues.

Whereas hyperirritability of the nervous system is an early manifestation of magnesium deficiency, marked passivity to ordinary stimuli was the rule at first in animals deprived of both calcium and magnesium. Indeed, in most of the dogs it persisted throughout the experimental period.

In some instances, however, this stability was eventually lost. After only 26 days on the diet deficient in magnesium and calcium, one animal suddenly succumbed to an attack of tonic-clonic convulsions which lasted 26 hours with only infrequent and short

intervals of abatement. The convulsions were characterized by lacrimal secretion, champing of the jaws, alternate flexion and extension of the extremities, and labored respiration which increased in rate as the seizures gained in severity. In this animal the serum calcium was 10.34 mg. per cent and the magnesium was 1.47 mg. per cent 3 days previous to the attack. Death by respiratory failure came 26 hours after the onset of hyperirritability.

Another dog survived 8 weeks without outward indications of hyperirritability before it was seized by tonic-clonic convulsions. This attack wore off in 3 to 4 hours. Thereafter the animal remained unable to stand, apparently due to weakness and spastic paralysis of the hind extremities. After 5 to 6 days, partial control of the hind legs was attained. The animal was in such an enfeebled condition, however, that a respiratory infection seemed imminent. Death occurred on the 8th day. Autopsy revealed a pulmonary congestion which might have been caused by aspiration during the earlier convulsions. In this dog the serum calcium was 7.32 mg. per cent and the serum magnesium was 0.63 mg. per cent 1 week before the attack.

As the survival period was prolonged, the remaining animals, still free from hyperirritability, ate progressively less. Instead of being alert and active, as was characteristic of their controls, they remained very quiet, indeed usually somnolent. The survival period of these animals, six in number, averaged 126 days and varied between 116 and 148 days (Table I). The final weeks were marked by extreme exhaustion. Death was without violence, except for one dog which died apparently in hypoglycemic convulsions.

In marked contrast to these frank symptoms of calcium-magnesium deficiency in young dogs, was the lack of manifestations in an older dog during a similar period of experimentation. This animal, an adult terrier (Dog 75), was subjected to the diet deficient in magnesium and calcium 177 days without exhibiting any of the typical symptoms. However, its serum calcium and magnesium diminished to subnormal values (Table II). In spite of the stress imposed by the rigorous exclusion of calcium and magnesium from the ration, the bones of the animal remained hard and well calcified, as judged by gross inspection.

*Chemical Changes in Blood*—A diminution in the magnesium

TABLE I  
*Effect of Calcium-Magnesium Deprivation on Food Consumption\**

Weekly food consumption of seven dogs deprived of calcium and magnesium in the diet compared with 4 dogs receiving the calcium-magnesium-deficient diet with added calcium and magnesium salts

Diet	Dog No	Wks on diet before obtaining food consumption data	Length of survival period	Diet consumed per wk											
				1	2	3	4	5	6	7	8	9	10	11	12
Calcium-magnesium-deficient	71-b ♀	9	wks	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm.
	72-c ♂	6	18	397	446	358	520	463	333	341	519	430	Dead		
	74-b ♂	10	9	537	300	167	Dead								
	75 ♀	14	20	392	373	361	409	274	423	376	310	140	91	Dead	
	77-b ♀	5	25	445	412	716	498	387	593	500	493	438	357	414	Killed
	78-b ♂	9	16	523	502	511	372	449	401	441	511	267	218	139	Dead
	81 ♂	8	19	583	512	600	600	450	760	776	500	430	190	Dead	
	73-b ♀	9	17	915	855	915	845	708	526	638	406	172	Dead		
Ca and Mg added	76-b ♂	8	16	725	710	845	920	1620	1870	1950	Killed				
	79-b ♀	9	19	1800	1965	2010	2100	2100	2050	1545	972	1113	463	849	Killed
		9	21	1800	2100	1967	1936	1300	1824	2050	1733	616	197	845	925 (Killed)
	82 ♀	8	17	1825	2065	2060	1700	2020	1630	†			Killed		

\* Food consumption records were kept only during the last 12 weeks of the studies.

† Food consumption records were not obtained due to scattering of food

content was the first change in the blood of the animals deprived of calcium and magnesium; in some cases the decrease during the 1st week amounted to 75 per cent (Chart 1). During the ensuing 4 to 5 weeks the magnesium values continued to decline progressively until they reached minimum values, at which level there-

TABLE II

*Effect of Calcium-Magnesium Deprivation on Calcium and Magnesium Content and Ca-Mg Ratio of Blood*

Summarized weekly values for dogs on the calcium-magnesium-deficient diet contrasted with those for control dogs receiving the same diet supplemented with calcium and magnesium salts

Diet	No of consecutive weekly determinations	Dog No	Ca, mg per 100 cc serum			Mg, mg per 100 cc serum			Ca Mg		
			Mean	Maximum	Minimum	Mean	Maximum	Minimum	Mean	Maximum	Minimum
Calcium-magnesium-deficient	15	71-b ♀	9 15	11 70	7 60	0 78	1 56	0 59	12 24	15 58	7 90
	8	72-c ♂	9 34	11 70	7 32	1 01	1 95	0 63	10 18	13 62	5 03
	14	74-b ♂	9 44	11 24	8 08	0 86	1 24	0 55	11 49	17 07	8 76
	18	75 ♀	9 03	11 75	7 07	0 82	1 30	0 42	11 62	19 59	9 18
	11	77-b ♀	9 15	11 18	8 20	0 86	1 08	0 67	10 75	12 27	9 44
	15	78-b ♂	8 70	10 65	7 28	0 72	1 15	0 52	12 48	14 83	9 26
	5	80 ♀	9 75	11 01	8 21	0 92	1 34	0 50	12 21	18 16	8 06
	14	81 ♂	9 65	12 22	7 70	0 74	1 51	0 43	13 90	18 18	7 36
Average			9 28	11 43	7 68	0 84	1 39	0 54	11 86	16 16	8 12
Ca and Mg added	15	73-b ♀	12 32	13 32	10 83	2 04	2 54	1 43	6 21	7 57	4 93
	19	76-b ♂	11 86	12 89	10 97	1 83	2 10	1 56	6 54	7 83	5 72
	15	79-b ♀	12 00	13 06	11 02	1 84	2 26	1 60	6 62	7 79	4 88
	14	82 ♀	12 11	13 48	11 02	1 78	2 11	1 56	6 84	7 57	6 08
Average .			12 07	13 19	10 96	1 87	2 25	1 54	6 55	7 69	5 40

after they remained essentially fixed. By the 5th week all the figures for magnesium, with one exception, were less than 1 mg. per cent of serum; afterwards the values remained between 0.6 and 0.8 per cent. In marked contrast to the lowering of serum magnesium in the dogs on the ration deficient in magnesium and calcium, the concentration of this element in the control dogs

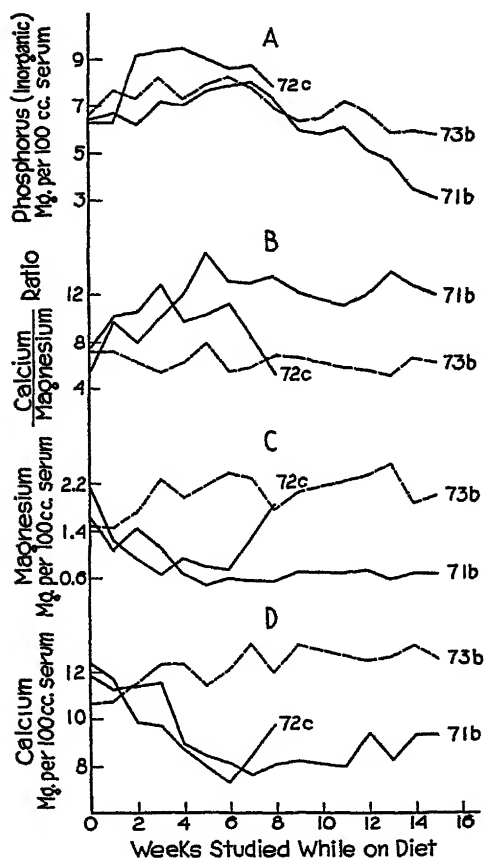


CHART 1. Changes in inorganic constituents of blood in dogs restricted to the calcium-magnesium-deficient diet, contrasted with values for a control dog on the calcium-magnesium-deficient ration with added calcium and magnesium salts. *D* represents the weekly figures for calcium; *C*, for magnesium; *B*, for the Ca Mg ratio as derived from the values found; and *A*, for inorganic phosphorus. In each case the curves with the solid lines indicate the values for animals deprived of calcium and magnesium; that with the broken line, values for the control dog receiving adequate calcium and magnesium in the diet. The numeral at the termination of each curve is the identification number of the dog.

throughout the experiment ranged between 1.78 and 2.04 mg. per cent with an average level of 1.84 per cent (Table II).

Unlike serum magnesium with its rapid decline, the serum calcium concentration did not decrease so readily in the experimental group, since initial values of 11 to 12 mg. per 100 cc. of serum prevailed in all animals, except one, for 3 to 4 weeks (Table II and Chart 1). After this lapse the values diminished to 10 mg. or less. In the exception to this trend (Dog 72-c) there was a drop to 9.9 mg. in the 2nd week; this animal, it should be noted, succumbed to the inadequate diet in only 9 weeks. Throughout the experimental period the control animals had serum calcium values averaging about 12 mg per cent. It is true that serum magnesium and serum calcium suffered decline in dogs deprived of these elements, but it should be noted that calcium underwent a less rapid and a less severe decline. Since the serum values for the two elements were not lowered to the same degree, the ratio of calcium to magnesium shifted. Specifically, the ratios for the four control dogs varied between 4.88 and 7.83, with an average of 6.55; in contrast, the figures for the experimental animals ranged between 5.03 and 19.59, with an average of 11.86 (Table II and Chart 1).

Among the inorganic constituents of blood affected by the calcium-magnesium deficiency were inorganic phosphorus of the serum and chlorides of whole blood. Usually serum phosphorus varies inversely with serum calcium, so that diminution of the latter in the experimental animals would seem to portend an elevation of phosphorus, or at least maintenance at a normal level. Actually, inorganic phosphorus did remain normal throughout almost the entire course of the deficiency, only to reach subnormal values in the terminal stages (Table III and Chart 1). The course of chlorides of whole blood, on the other hand, was different. Although serum sodium remained unchanged after the diet deficient in magnesium and calcium, chloride values mounted significantly (Table III), owing perhaps to the alterations in red cell volume. As the deficiency was prolonged the red blood cell volume suffered a decrease. For example, the mean red cell volume of a control dog was 46, but it was only 37 and 38, respectively, for two dogs deprived of calcium and magnesium (Table III).

In dogs deprived only of magnesium there is no indication of diminished alkali reserve (21). In contrast, the animals subjected to magnesium-calcium deficiency suffered a slight, but significant, lowering in the carbon dioxide capacity and pH of the plasma

TABLE III

*Effect of Calcium-Magnesium Deprivation on Inorganic Constituents of Blood*

Weekly values for Dogs 71-b ♀ and 72-c ♂ on the calcium-magnesium-deficient diet contrasted with those of a control, Dog 73-b ♀, on the calcium-magnesium-deficient diet with added calcium and magnesium salts

Wks studied while on diet	Weight, kilos			R b c, vol per cent			P (inorganic), mg per 100 cc serum			K, mg per 100 cc serum			Na, mg per 100 cc serum			Cl (as NaCl), mg per 100 cc blood		
	Dog 73-b	Dog 71-b	Dog 72-c	Dog 73-b	Dog 71-b	Dog 72-c	Dog 73-b	Dog 71-b	Dog 72-c	Dog 73-b	Dog 71-b	Dog 72-c	Dog 73-b	Dog 71-b	Dog 72-c	Dog 73-b	Dog 71-b	Dog 72-c
Preliminary	2 95	2 84	2 39	34		38	6 7	6 5	5 6	25 7	23 7	26 6	323	323	308	494	494	486
1	2 95	2 62	2 62	35	36	35	7 7	6 9	6 3	25 5	26 1	21 6	317	306	307	510	503	524
2	3 29	2 84	2 84	35	34	36	7 4	6 2	9 2	25 9		26 4	307	306	315	494	515	486
3	4 32	3 18	3 07	37	35	43	8 6	7 5	9 4	27 0	24 4	21 3	313	318	325	469	492	481
4	5 00	3 64	3 29	38	39	38	7 1	7 1	9 7	22 0	22 5	23 0	331	315	336	505	494	475
5	5 34	3 75	3 40	38	41	40	7 9	7 9		22 3	22 8		305	329		494	484	
6	5 91	3 75	3 40	46	44	42	8 1	8 2	8 1	23 8	22 6	18 6	327	327	331	480	488	472
7	6 47	3 86	3 52	46	40	41	7 7	8 1	8 9	22 7	23 4	26 6	342	324	349	477	497	504
8	6 82	3 75	3 07	45	38	32			7 6			27 7			348			535
9	6 82	3 52		50	40		6 4	6 3		19 9	18 5		315	327		469	508	
10	7 05	3 40		50	37		6 6	5 9		19 2	19 1		319	320		495	501	
11	7 05	3 40		54	36		7 3	6 5		19 7	22 1		324	327		469	539	
12	7 27	3 29		60	40		6 8	5 2		21 6	22 4		322	321		472	529	
13	7 05	3 18		52	34		5 8	4 9		19 9	21 8		330	335		471	527	
14	8 18	3 18		48	33		5 9	3 7		21 2	24 3		340	322		483	550	
15	8 97	3 07		56	30		5 6	3 3		19 4	22 2		318	323		467	561	
Mean	6 17	3 36	3 15	46	37	38	7 1	6 3	8 5	22 2	22 5	23 6	322	321	330	482	513	497
Maximum	8 97	3 86	3 52	60	44	43	8 6	8 2	9 7	27 0	26 1	27 7	342	335	349	510	561	535
Minimum	2 95	2 62	2 62	35	30	32	5 6	3 3	6 3	19 2	18 5	18 6	305	306	307	467	484	472



(Table IV). Although Table IV does not indicate the rate of change, it may be stated that the values for carbon dioxide-combining power follow the general trend of those for serum calcium (Chart 1). An average of the values for carbon dioxide capacity during the entire period of calcium-magnesium deficiency amounted to 41.9 and 41.4 volumes per cent, respectively, for Dogs 81 and 80, the average was 44.7 volumes per cent for the control, Dog 82. This diminution in alkali reserve paralleled the

TABLE IV

*Effect of Calcium-Magnesium Deprivation on Alkali Reserve and Plasma Proteins of Blood*

Values for Dogs 81 ♂ and 80 ♀ on the calcium-magnesium-deficient diet compared with those of a control, Dog 82 ♀, on the calcium-magnesium-deficient diet with added calcium and magnesium salts. These values were obtained by weekly determinations extending over 14 weeks in the case of Dogs 82 and 81 and 5 weeks in the case of Dog 80

	Weight, kilos			R b c, vol per cent of plasma			CO <sub>2</sub> capacity, vol per cent of plasma			pH of plasma		
	Dog 82	Dog 81	Dog 80	Dog 82	Dog 81	Dog 80	Dog 82	Dog 81	Dog 80	Dog 82	Dog 81	Dog 80
Mean . . .	8 33	4 60	3 38	41	35	37	44 7	41 9	41 4	7 53	7 48	7 50
Maximum	10 90	5 23	4 09	52	45	42	52 2	52 2	42 8	7 58	7 60	7 54
Minimum	4 55	2 95	2 50	32	30	32	39 0	33 4	39 0	7 48	7 39	7 46
	Fibrinogen, per cent			Albumin, per cent			Globulin, per cent			Total protein, per cent		
	Dog 82	Dog 81	Dog 80	Dog 82	Dog 81	Dog 80	Dog 82	Dog 81	Dog 80	Dog 82	Dog 81	Dog 80
Mean	0 26	0 52	0 42	4 71	4 27	4 28	1 35	2 02	1 44	6 32	6 81	6 09
Maximum	0 38	0 73	0 51	5 23	4 73	4 47	1 81	3 25	1 72	7 07	8 44	6 43
Minimum	0 20	0 36	0 37	4 15	3 94	3 92	0 94	1 19	1 25	5 60	5 61	5 72

values for plasma pH of the experimental animals. For Dogs 81 and 80, the average figures for pH were 7.48 and 7.50, respectively; whereas the value for the control dog was 7.53 (Table IV). It should be emphasized that these averages for both carbon dioxide capacity and pH represent the entire period of the deficiency; therefore they are debased and made less divergent by inclusion of data for the first 4 weeks on the deficient diet, when no marked difference existed between the control and the experimental animals

The blood lipids in magnesium deficiency show unusual alterations which have been correlated with the onset of nutritive failure; namely, total cholesterol increases markedly, due to high ester values; fatty acids decrease commensurately; lipid phosphorus remains normal (21). This blood picture was not duplicated in cases with magnesium-calcium deficiency. In fact, examination

### Effect of Calcium-Magnesium Deprivation on Blood Lipids

Blood lipid values in a control, Dog 79-b ♀, contrasted with those in Dogs 78-b ♂ and 77-b ♀ deprived of calcium and magnesium in the diet during (a) the first 4 weeks of the deficiency and (b) the remaining 11 weeks of the 16 weeks survival period of Dog 77-b and 11 weeks of the 19 weeks survival period of Dog 78-b. The figures represent the mean of values obtained weekly during the periods indicated.

Period	Total lipid per 100 cc plasma			Fatty acids per 100 cc plasma			Cholesterol per 100 cc plasma			Cholesterol esters per 100 cc plasma								
	Dog 79-b	Dog 78-b	Dog 77-b	Dog 79-b	Dog 78-b	Dog 77-b	Dog 79-b	Dog 78-b	Dog 77-b	Dog 79-b	Dog 78-b	Dog 77-b						
1st 4 wks on diet	456	466	219	289	302	125	167	164	88	112	124	56						
5th wk on diet to termination of study	518	516	464	345	362	328	172	155	135	107	107	75						
	Free cholesterol per 100 cc plasma			Esters as per cent of total cholesterol			Cholesterol esters Free cholesterol			Lipoid P per 100 cc blood								
	mg	mg	mg							mg.	mg	mg						
1st 4 wks on diet	55	40	32	67	175	63	62	043	101	75	14	0	15	2	15	5		
5th wk on diet to termination of study	65	48	60	62	269	055	61	65	2	23	1	25	15	7	15	0	14	7

of Table V and Chart 2 reveals no indication of any significant disturbance in the blood lipids of experimental Dog 78-b, for its values are in excellent accord with those of the control animal, Dog 79-b. Unfortunately, experimental Dog 77-b contracted a respiratory infection during the 1st week of the experiment and did not recover until the 4th week. During this period its values for total lipids, fatty acids, total cholesterol, and cholesterol esters

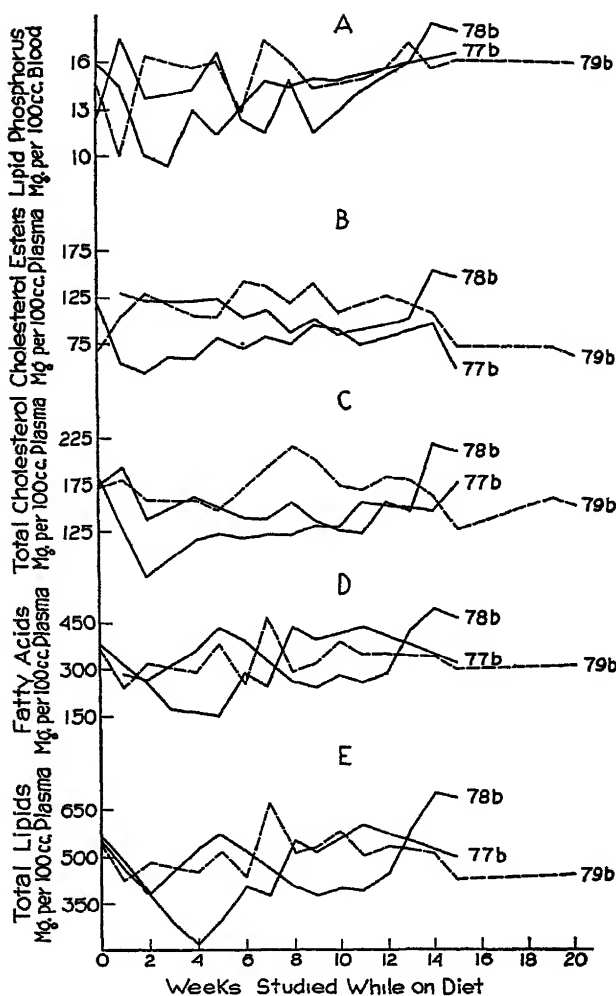


CHART 2 Values for lipid constituents of blood of dogs restricted to the calcium-magnesium-deficient diet, contrasted with values for a control dog on the same diet with added calcium and magnesium salts. *E* represents the weekly values for total lipids (fatty acids plus cholesterol); *D*, for fatty acids; *C*, for total cholesterol; *B*, for cholesterol esters; and *A*, for lipid phosphorus. In each case the curves with the solid lines indicate the values for animals deprived of calcium and magnesium, that with the broken line, values for the control dog receiving adequate calcium and magnesium in the diet. The numeral at the termination of each curve is the identification number of the dog.

were abnormally low; thereafter they rose. Table V thus appears to show for this dog a late increase in all lipid constituents, but it is only the expected rise to the normal level, for the values from the 5th week until death were entirely within normal limits as cited by Glusker (27) and by Bloor (28). Thus, no significant changes in lipid metabolism occurred as the result of calcium-magnesium deprivation.

TABLE VI

*Effect of Calcium-Magnesium Deprivation on Glucose and Non-Protein Nitrogenous Constituents of Blood*

Values for Dogs 75 ♀ and 74-b ♂ on the calcium-magnesium-deficient diet compared with those for a control, Dog 76-b ♂, on the calcium-magnesium-deficient diet with added calcium and magnesium salts. These values were obtained by weekly determinations extending over 25 weeks in the case of Dog 75 and 20 weeks in the case of Dog 74-b.

	Weight			R b c, vol per cent			Glucose per 100 cc blood			Non-protein N per 100 cc blood		
	Dog 76-b	Dog 75	Dog 74-b	Dog 76-b	Dog 75	Dog 74-b	Dog 76-b	Dog 75	Dog 74-b	Dog 76-b	Dog 75	Dog 74-b
	kg	kg	kg				mg	mg	mg	mg	mg	mg
Mean	6.86	2.85	3.23	39	38	45	92	91	88	27	28	28
Maximum	9.43	3.29	3.52	48	49	53	105	111	100	32	33	48
Minimum	3.52	2.73	2.39	31	30	36	75	80	42	25	24	24

	Creatine and creatinine per 100 cc blood			Preformed creatinine per 100 cc blood			Creatine per 100 cc blood		
	Dog 76-b	Dog 75	Dog 74-b	Dog 76-b	Dog 75	Dog 74-b	Dog 76-b	Dog 75	Dog 74-b
	mg	mg	mg	mg	mg	mg	mg	mg	mg
Mean	5.1	5.0	5.1	1.2	1.3	1.2	3.8	3.8	3.9
Maximum	5.6	5.9	5.9	1.3	1.4	1.4	4.6	4.6	4.8
Minimum	4.2	4.1	4.0	1.0	1.1	1.1	3.0	2.9	2.8

The terminal stages of the magnesium-calcium deficiency, i.e. the last week or two of the survival period, were marked by a rise in non-protein nitrogen (Table VI), which was of the same magnitude as that reported in magnesium deficiency. The blood sugar remained normal except in the terminal stages, when it diminished. Dog 74-b had only 42 mg. per cent of glucose 6 hours before death in hypoglycemic convulsions.

Of the plasma proteins, fibrinogen rose to values approximately

50 per cent above normal (Table IV) during the latter stages of the deficiency. Plasma albumin and globulin remained normal.

No changes were detected in the plasma bile pigment

#### DISCUSSION

Before discussing the symptoms which ensue from the diet lacking at once both calcium and magnesium, and whatever implications may be gathered from them for a physiological antagonism, it might be advantageous to consider the effects of deprivation of each element separately, those of calcium deficiency in contrast to those of magnesium deficiency. Of the phenomena following upon a restricted intake of calcium, it is difficult, for two reasons, to give a clear cut portrayal. First, since the concept of an adequate diet was, as we now know, inexact and incomplete at the time most of the studies were conducted (29-33), the rations used then were deficient in several respects, particularly the vitamins, therefore, the experiments are not now susceptible to rigid interpretation. Secondly, failure of appetite, as a complicating feature in calcium deficiency, introduces a restricted intake, not of calcium alone, but of all dietary components; this involvement by partial starvation, with a symptomatology of its own, clouds the course of the calcium deficiency. Nevertheless we shall present those symptoms which have been displayed by animals on low calcium rations and which, through exclusion of possible effects of other dietary factors, may be presumably attributed solely to lack of calcium. They are gastrointestinal attacks characterized by foul, bloody diarrhea, progressive anorexia, loss of weight (34), epiphora, bone resorption, and hyperirritability of the nervous system, often eventuating in tonic-clonic convulsions. At least two of these symptoms, indicative of alteration in both bone composition and in neuromuscular response, deserve more than casual mention.

As judged by chemical and especially by microscopic examination, the bone changes resulting from calcium deficiency may assume now the form of osteoporosis, now that of rickets; between these two extremes the pathological picture may partake of all intermediary gradations and complex combinations (24, 25, 35, 36). This variability in effect may be readily understood when it is remembered that the bone is sensitive to the relative proportions of calcium and phosphorus, and the presence of vitamin D or

sunlight (37). When these factors concerned in the development of bone are imperfectly balanced, they determine whether osteoid tissue shall be formed in slight or in lavish amount, whether the bone elements shall be distributed in a regular or disorderly state, whether calcium deposition shall be induced or checked, or even be supplanted by resorption; in short, whether the changes in bone morphology shall be directed towards osteoporosis or rickets.

In affecting the nervous system, calcium deficiency brings on hyperexcitability with tonic-clonic convulsions, the symptom complex designated as tetany. Loeb (4), from the reactions of isolated muscle-nerve preparations, indicated that the irritability of the nervous system is under the sway of four elements: sodium, potassium, magnesium, and calcium. The lowered blood calcium, demonstrated later in parathyroid tetany (38) and in infantile tetany (39), fell into agreement with this view. As significance became attached in due course to ionic calcium in the blood, as the factors influencing its concentration were determined, and as conditions favorable to lowering the concentration were recognized in the several forms of tetany, both old and new, the tendency, now known to be misdirected, was to attribute all forms of tetany to disturbed blood calcium. Despite the similarity in lowered calcium level of the blood, the syndrome of calcium deprivation in animals differs from infantile tetany, the most common clinical form of tetany, in its mode of onset and in the pattern of hyperirritability and convulsions. Perhaps the difference is explained by the rate of change in blood calcium level in the two conditions: calcium deficiency is characterized by a gradual depletion of the calcium stores in the body and by the slow but progressive lowering of the blood calcium, infantile tetany, on the contrary, is characterized by the abruptness of a downward shift in blood calcium in a setting of preexisting rickets, a kaleidoscopic turn of events that has been successfully simulated in animals (40-45). Just as plausibly the difference might be due to anorexia which ushers in partial inanition with its own distinctive symptomatology, for consequent loss of weight is usually conspicuous in calcium deficiency and animals not infrequently die from the nutritive failure rather than from convulsions.

Like calcium deficiency, magnesium deprivation acts predominantly upon the osseous and nervous systems, yet the effects in

the two conditions bear no resemblance. An initial hypercalcification of the bones, due to calcium retention, which is the immediate response to magnesium deficiency, later gives way to normal deposition of calcium as calcium excretion resumes (1, 2). The nervous symptoms, hyperirritability, vasodilatation, and tonic-clonic convulsions, which comprise magnesium tetany, represent a syndrome which is separate and distinct from other forms of tetany (20, 23). Vasodilatation imparts a florid appearance to such skin areas as are readily visible, particularly the inner aspect of the ears, the nail beds, tongue, conjunctiva, and buccal mucosa (22). The ears, at first swollen and spongy, later become thick, leathery, and mottled. Hyperexcitability, particularly to auditory stimuli, increases until it is so pronounced that any undue noise may release convulsive seizures, tonic and clonic in character. Successive convulsive attacks are punctuated by brief intermissions for recovery from exhaustion. In such an interval, the animal, while apparently on the way to recovery, usually succumbs. Appetite, as judged by food intake, is little altered throughout. If the survival period is sufficiently prolonged, nutritive failure appears with slight decline in weight. Regardless whether nutritive failure has set in, convulsions always carry off the animals. With these symptoms of magnesium tetany may be correlated the blood changes; serum magnesium undergoes a prompt and rapid decline until it reaches an extremely low level where it usually remains stationary. On the other hand, calcium, phosphorus, pH, carbon dioxide capacity, and chlorides yield only normal values.

With these two syndromes in mind, it is not difficult to ascertain points of similarity and difference which the magnesium-calcium deficiency bears to each of them. In animals deprived of both calcium and magnesium, there appeared gastrointestinal disturbances in which prolonged obstipation was followed by severe diarrhea with copious discharges of mucous, blood-flecked fluid. At the same time, progressive anorexia set in, whereby diminished intake of food led ultimately to decline in weight. In the bones of those animals with sufficiently long survival periods, there was marked osteoporosis as evidenced by resorption. In reference to the nervous system, two animals were hyperirritable: one died in convulsions; the other developed transient spastic paralysis of the

hind extremities following a convulsive attack. What is more important, however, the other six animals were not even active and alert, but quiet and somnolent. The survival periods of these dogs subjected to a diet with a magnesium-calcium deficiency were much more protracted than those of animals on a magnesium-deficient diet. Thus the symptoms of magnesium-calcium deficiency, diarrhea, anorexia, nutritive failure, osteoporosis, marked passivity to stimuli, and prolonged survival period, partake predominantly of a calcium deficiency, to the exclusion of those characteristics peculiar to magnesium deficiency. Similarly, the blood changes of calcium-magnesium deprivation, in the main, do not adhere to those of magnesium deficiency. True, both show diminished serum magnesium, but the decrease in calcium, phosphorus, pH, and carbon dioxide capacity, and the increase in fibrinogen (46) and chlorides, displayed in the magnesium-calcium deficiency, are changes not seen in magnesium deficiency and probably are characteristic of calcium deficiency. Thus both in symptomatology and blood changes, the magnesium-calcium deficiency resembles, qualitatively at least, calcium deficiency, with little that is characteristic of magnesium deficiency.

Concerning a physiological antagonism between calcium and magnesium, the evidence drawn from the effects of an excess or of an inadequacy of one element upon the other, as revealed by bone composition or metabolic data, has been cited (1, 2). For judgment of the operation of an antagonism in the present study on the magnesium-calcium deficiency, we are limited to consideration of symptoms and blood data. When either calcium or magnesium in the blood is sufficiently low, the result is tetany. If deprivation of either element singly can bring about this condition, it would be expected that a diminution in the blood level of both elements, occasioned by the magnesium-calcium dietary deficiency, would lead to a more rapid onset and a more severe course of tetany. Consider the swift, stormy sequence of events in magnesium deficiency, with its early hyperirritability, its comparatively short survival period, and its violently fatal convulsions. Any tetany that calcium-magnesium deprivation would induce might be expected to transcend even that of low magnesium. But the symptomatology does not bear out this expectation. No hyperexcitability is evident in the animals deprived of both calcium and



magnesium at a time when animals deprived of only the latter element would have already manifested marked changes. Indeed it is significant that, in the magnesium-calcium deficiency, a long, completely passive survival period with a non-convulsive death is the rule. If tetany does appear, it is more like that of the low calcium type. Perhaps the outstanding feature of calcium-magnesium deprivation is the complete obliteration of the rapidly moving and fatal tetany characteristic of lack of magnesium. Tetany had not appeared when changes in the blood calcium and magnesium were indicative of it. Whether lessened alkalinity in the blood or anorexia has contributed to the mechanism is unknown. In any event, it is evident that simultaneous deprivation of calcium and magnesium has operated, not in intensifying the severity of tetany, but in repressing it to the point of extinction. The lack of calcium has so counterbalanced the lack of magnesium that spectacular nervous symptoms scarcely ever occur. These data offer additional evidence in favor of the so called physiological antagonism of calcium towards magnesium.

Finally, although representative blood constituents are so unaltered as to provide no clews, symptoms reveal that nutritive failure is more pronounced in calcium-magnesium deprivation than in magnesium deficiency alone. The onset of fatal convulsions in the magnesium syndrome, it will be remembered, prevented nutritive failure from exerting its full effect. However, the intervention of partial starvation into the course of calcium-magnesium deficiency acts to make nutritive failure in it a more gradual and prolonged process, responsible even for death. On the part of blood chemistry, magnesium deficiency did show significant changes, for there the course of nutritive failure was correlated with concomitant alterations in the level of the blood lipids. After serum magnesium had reached its low point, total cholesterol underwent a marked increase, fatty acids, on the contrary, suffered a commensurate decrease, so that total fat, the calculated sum of cholesterol and fatty acids, remained constant. Since the increase in total cholesterol was due largely to the rise in values of its ester fraction, the percentage of total cholesterol in the ester form was unusually high. In calcium-magnesium deficiency, these constituents did not show this behavior. Compared with control as well as published normal values, with due allowance for

age and diet (27, 28), none of the blood lipids suffered any change as the result of the magnesium-calcium deficiency. It is probable that partial starvation imparts low lipid values to calcium deficiency, a circumstance which, in the magnesium-calcium deficiency, effectively offsets the peak values accompanying magnesium deprivation. This explanation lies, of course, in the realm of pure conjecture. Certainly calcium deprivation with its inevitable inanition, when superimposed upon magnesium restriction, modifies the course of nutritive failure and its associated blood changes as they are seen in magnesium deficiency.

#### SUMMARY

The symptomatology and blood chemical changes have been observed in young dogs receiving a diet deficient in both calcium and magnesium, but otherwise adequate. The dietary deficiency is manifested by edema, gastrointestinal disturbance with alternate constipation and diarrhea, osteoporosis with consequent deformation of the bones, in a few cases by hyperirritability of the nervous system, which led to fatal convulsions, but in the majority of cases by a marked inertness, and anorexia which usually led to death by consequent inanition. The blood chemical data, obtained by weekly analysis throughout the survival period, reveal an immediate decrease in serum magnesium quantitatively similar to that which induces tetany in magnesium deprivation. Serum calcium likewise diminished to levels usually indicative of tetany. Alkali reserve and pH diminished; blood chlorides mounted as the erythrocyte volume fell; fibrinogen increased; terminally inorganic phosphorus decreased and non-protein nitrogen rose. Significant changes did not appear in serum sodium and potassium, plasma total lipids, fatty acids, total cholesterol, cholesterol esters, lipid phosphorus, serum bile pigment, and plasma albumin and globulin.

In comparing the effects of the calcium-magnesium deprivation on the one hand with those of calcium and magnesium deficiencies individually on the other hand, it was evident that the deficiency in both resembled more closely the low calcium syndrome. From symptoms and blood data it was possible to consider the so called physiological antagonism between calcium and magnesium. It was pointed out that simultaneous diminution of serum calcium prevented symptoms of magnesium tetany as serum magnesium

reached abnormally low levels. Finally, it was determined that, on the basis of symptoms, nutritive failure was more pronounced in the magnesium-calcium deficiency than in magnesium restriction alone.

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## DERIVATIVES OF KERATIN

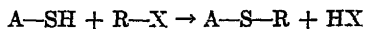
BY DAVID R. GODDARD\* AND LEONOR MICHAELIS

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York)

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In an earlier paper the authors (1) expressed the view that keratins are fibrous proteins whose characteristic properties are essentially determined by the S—S groups of cystine which act as very firmly established cross links uniting the elementary fibers of polypeptide chains. This view was based on the action of certain alkaline reductants which could be shown to reduce disulfides and convert keratin into an amorphous protein soluble in weak alkalis, digestible by true proteases, and in which the sulfur is in the sulfhydryl state. The important rôle of the disulfide bond had already been emphasized by Speakman and Huist (2) and Astbury (3) on the basis of their chemical and physical studies, including x-ray diffraction patterns of keratins.

Since keratins have a very high percentage of disulfide sulfur (10 to 15 per cent cystine) and may readily be reduced to sulfhydryl proteins by alkaline thioglycolate, we realized that this reduced protein might be a useful material for study as an example of a sulfhydryl protein. We were particularly interested in studying the properties of derived proteins formed by substitution in the sulfhydryl group by reaction with organic halogen compounds according to the following scheme.



This reaction occurs with great ease at neutral to mildly alkaline reaction, and has already been applied to proteins for different reasons by Mirsky and Anson (4) and Goddard and Schubert (5). Although organic halogen compounds can react with amino groups according to Michaelis and Schubert (6), we will show that it has been possible under certain conditions to substitute completely

\* National Research Fellow in the Biological Sciences.

the sulfhydryl groups of the protein of reduced wool keratin, without substituting amino groups.

The proteins studied are the sulfhydryl protein obtained by the alkaline thioglycolate reduction of wool, this protein after it has been reoxidized to the disulfide form, and the substituted proteins formed by reaction of the sulfhydryl protein with iodoacetic acid, iodoacetamide, iodoethyl alcohol, and  $\alpha$ -bromopropionic acid, at mildly alkaline reaction. The disulfide protein may be formed by reoxidation of the sulfhydryl protein at neutral reaction by the air or ferricyanide. As to the nomenclature of these proteins, we may designate the reduced keratin as kerateine, and the reoxidized, amorphous kerateine as metakeratin. By reaction of kerateine with iodoacetate we obtained a carboxymethylkerateine; with  $\alpha$ -bromopropionic acid,  $\alpha$ -carboxyethylkerateine; with iodoethyl alcohol, hydroxyethylkerateine; with iodoacetamide, carbamyl-methylkerateine.

None of the derived keratins has been obtained in a strictly pure state, nor may they be considered as chemical entities. It is not even likely that the keratin of a single hair is a homogeneous chemical substance. Furthermore, it should be borne in mind that the reduction of the disulfide group by thioglycolate is a reversible reaction, and its completeness depends on the excess of the reductant applied, and that the reaction with the halogen compounds can proceed only to that extent to which the S—S group has been previously reduced. In the preparation of a substituted protein the non-reduced, non-substituted residue can be determined as cystine by applying the Folin-Marenzi method to the hydrolysate. The substituted sulfur does not react in this method.

The analytical data for these proteins and wool are presented in Table I. It will be seen that the S and N are similar to the values of wool, and secondly, that nearly all the cystine has been reduced and substituted.

Proceeding to the isoelectric points and solubilities of these derived proteins, it should be interesting to compare native wool with the derived proteins in this respect. There is no agreement as to the isoelectric point of native wool Elöd and Silva (7), from swelling experiments and changes in the pH of solutions in which wool was allowed to come to equilibrium, determined the isoelectric point to be 4.9. This is in agreement with the recent result

TABLE I  
Analysis of Keratin Derivatives (Per Cent, Except Column of Isoelectric Points)

Protein	Total S	Total N	Cystine	Cysteine S	Amino N	Isoelectric point	Solubility
Native wool	3 35 3 33	16 29	11 6	3 12		4 9(?)	Insoluble in M NaOH or HCl
Keratene	3 34	16 50	12 2*	3 24		4 6-4 9	Soluble in 0.1 M Na <sub>2</sub> CO <sub>3</sub> and NH <sub>4</sub> OH; insoluble in M/30 HCl
Metakeratin	3 37	16 50 16 54	12 2, 12 1	3 25	0 81, 0 79	4 5-4 7	Soluble in 0.1 M Na <sub>2</sub> CO <sub>3</sub> and NH <sub>4</sub> OH; insoluble in M/30 HCl
Carboxymethylkeratene, unfractionated†	3 31		1 42	0 38		3 8-4 3	Soluble in 0.1 M sodium acetate or M/30 HCl
Carboxymethylkeratene, Fraction A	3 33	16 32	0 80	0 21	0 81, 0 78	3 7-4 3	Soluble in 0.1 M sodium acetate and M/30 HCl
Carboxymethylkeratene, Fraction B	3 29 4 55	14 95	0 97	0 26	0 50	3 0-3 3	Soluble in 0.1 M sodium lactate and lactic acid
Carboxymethylkeratene‡	2 00	15 90	1 73	0 46	0 80, 0 76	3 3-3 6	Soluble in 0.1 M sodium acetate and M/30 HCl
Carbamylmethylkeratene	2 78 2 84	17 35§	2 74 2 40	0 75		5 0-5 3	Insoluble in sodium acetate; soluble in M/30 HCl
$\alpha$ -Carboxyethylkeratene	2 12 2 15 2 17	15 84	1 35	0 36	0 76	3 1-3 7	Soluble in 0.1 M sodium acetate and M/30 HCl
Hydroxyethylkeratene	2 73 2 76 2 77	15 50 15 30	2 66	0 71		4 5-4 9	Soluble in 0.1 M Na <sub>2</sub> CO <sub>3</sub> or NH <sub>4</sub> OH, insoluble in M/30 HCl or 0.1 M sodium acetate

\* Total cystine = 12.2; of this 88 per cent was in the form of cysteine.

† This was not the preparation from which Fractions A and B were prepared.

‡ This preparation was allowed to react with iodoacetate for 40 hours at pH 9.0 to 9.5. Notice the conspicuous loss of sulfur.

§ The increased N content is due to the amide N of iodoacetamide



of Dumanski and Dumanski (8) of 4.9 obtained by deflections of wool fibers in an electrical field. Speakman (2) denies that wool has any distinct isoelectric point and acknowledges only a wide isoelectric zone of 5.0 to 7.0. Harris (9) has determined the isoelectric point of wool as 3.4 by cataphoresis of wool particles. This disagreement makes the comparison with the derived proteins difficult. In these derived proteins there is no difficulty in determining the isoelectric point with the flocculation method (10). Kerateine and metakeratin prepared from wool have isoelectric points in the region 4.6 to 4.7. Carboxymethylkerateine and  $\alpha$ -carboxyethylkerateine have much lower isoelectric points, as would be expected from introduction of the carboxyl groups. Carbamylmethylkerateine has a slightly higher isoelectric point, while hydroxyethylkerateine has the same isoelectric point as kerateine. The change in isoelectric points and solubilities of these derived proteins are what would be expected from the chemical nature of the substituted groups.

A comparison of the solubilities of these compounds shows the following features. Wool is insoluble in all acids and bases except in so far as it is hydrolyzed. Wool dissolves readily in alkaline solutions of NaCN, Na<sub>2</sub>S, and thioglycolate (1), but in all these cases the fibrous pattern is irreversibly destroyed. Metakeratin and kerateine of wool are dissolved readily in weak (0.1 M) NaOH, Na<sub>2</sub>CO<sub>3</sub>, and NH<sub>4</sub>OH. They are insoluble in sodium acetate, water, and neutral salts, and slightly soluble in dilute HCl. Stable neutral solutions containing several gm. of metakeratin in 100 cc. may be obtained by dialyzing the alkaline solution. These solutions are precipitated by traces of acetic or other acids, and once precipitated are as difficultly soluble as the undialyzed metakeratin. Carboxymethylkerateine and carboxyethylkerateine are very readily soluble not only in solutions of NaHCO<sub>3</sub> and dilute HCl, but even in a solution of sodium acetate. Carbamylmethylkerateine has the same solubility in alkalies as kerateine, but is much more soluble in dilute HCl than is kerateine. The solubility of hydroxyethylkerateine is similar to kerateine.

Determinations of free amino N of some of our preparations were performed by the method of Van Slyke (11) in the constant volume apparatus. The main purpose of this analysis was to show whether under the conditions of our work a substitution of the H

atoms of the amino group occurred by reaction of kerateine with iodoacetate, which could be imagined according to the results of Michaelis and Schubert with simple amino acids (6). The figures show definitely that no detectable substitution of amino groups occurred, even in a period of 40 hours at a pH about 9.0 to 9.5. This is of interest to the general problem as to the point of attack of iodoacetate on the enzymes which it poisons.

As regards the percentage of amino nitrogen, Van Slyke and Birchard (12) found that in several proteins the free amino N was equal to half the lysine nitrogen, with the exception of gliadin in which it is much higher. The free amino N, as determined by the Van Slyke method on metakeratin and several derived kerateines, is somewhat higher than would be expected from the published values of the lysine content of wool of 2.2 (13), 2.3 (14), and 2.8 (15) per cent. If we take the nitrogen content of wool as 16.5, then the percentage of free amino nitrogen referred to total nitrogen should be 1.8 to 2.3 per cent, depending upon the value assumed to be the correct lysine value. We find that 4.7 to 4.9 per cent of the total N is free amino N in our preparations.

It is not likely that the whole substance of a hair, even disregarding any pigment, represents a single homogeneous protein. The usual method of separating a natural protein mixture into its component parts is the fractional precipitation with salts. Such a method obviously cannot be used for an insoluble protein such as keratin. Even for kerateine and metakeratin the solubility is too low for an attempt of fractionation. But the much wider range of solubility of some of the derived proteins makes possible fractional precipitation. Carboxymethylkerateine was fractionated with ammonium sulfate from solution of the protein in 0.1 M sodium acetate. The fractionation led to two fractions; one which contained the major bulk of the original protein and had a similar content of S, N, and amino N as the original protein; and a second small fraction, Fraction B, which showed higher S and lower N and amino N content. Fraction A is completely precipitated from solution by 35 per cent saturation with  $(\text{NH}_4)_2\text{SO}_4$  and gives a compact, cheese-like precipitate. This fraction is completely insoluble at its isoelectric point, it does not dissolve appreciably in dilute lactic acid or sodium lactate, but does dissolve readily in dilute sodium acetate or dilute HCl. The other

fraction, Fraction B, is soluble in 35 per cent saturated  $(\text{NH}_4)_2\text{SO}_4$ , but is completely precipitated as a very sticky, non-flocculent precipitate at 60 per cent saturation. It is not completely insoluble even at its isoelectric point and readily dissolves in an excess of dilute lactic acid or even in sodium lactate. The high sulfur content of Fraction B indicates that the native keratin probably consists of two fractions, and not that the substitution leads to two different products

One of the characteristic properties of keratin is its complete resistance to digestion by trypsin and pepsin. But all of the proteins prepared from wool, either as sulfhydryl, disulfide, or substituted proteins, are as readily digested by trypsin and pepsin as are typical proteins. The resistance of keratin to digestion is not dependent upon the disulfide state as such, since metakeratin is readily digested, but upon the fibrous property of the keratin, as brought about by the spacial arrangement of the S—S bonds. Once this bond has been broken, the protein becomes digestible by true proteases no matter what other reactions the sulfur may undergo.

As regards the oxidation and reduction of S of these proteins, native keratin may only be reduced at a pH of 10 or higher, but metakeratin is reduced by the same agents at a pH of 7 to 8. Kerateine is readily oxidized to metakeratin, even dialysis of the protein suspended in acetate buffer at pH 4.6 leads to nearly complete oxidation in 3 days. In an alkaline solution oxidation is much more rapid. Within the short time required to dissolve kerateine in ammonia, to filter, and to reprecipitate (20 to 30 minutes), complete oxidation takes place. Such a high degree of autoxidizibility is as a rule not encountered in other sulfhydryl proteins according to A. E. Mirsky (personal communication).

#### EXPERIMENTAL

*The "Parent" Protein*—For the preparation of the substituted proteins a single batch of wool protein, essentially metakeratin still containing some unoxidized kerateine, was used. This protein is spoken of as the "parent" protein. It was prepared essentially as described by Goddard and Michaelis (1). 100 gm of defatted but otherwise native, untreated wool are dissolved by mechanical shaking in 2 liters of 0.5 M disodium thioglycolate (if

the pH is maintained between 11 to 12, this takes about 3 hours). The undissolved residue is removed by centrifugation followed by filtration, the filtrate is precipitated with acetic acid, collected on the centrifuge, washed with acetone, and ground in a mortar with acetone. After freeing from acetone by a vacuum, the protein suspension is dialyzed in cellophane tubes until free of salts and thioglycolic acid. The dialyzed protein is washed with acetone and dried in a vacuum desiccator. It is obtained as a fine white powder containing about 4 per cent moisture, nearly insoluble in water, but slightly soluble in acids, and very (but slowly) soluble in alkali. This powder has essentially the same content of nitrogen, sulfur, and cystine as the wool from which it is prepared. It becomes to a great extent reoxidized on preparation. A typical preparation, hydrolyzed and analyzed by the Mirsky and Anson (4) modification of the Folin-Marenzi method, contained 12.3 per cent of total cystine (cystine + cysteine) and 3.17 per cent of cysteine. So, the sulfur, which had been practically completely reduced, has been reoxidized during the preparation to 75 per cent.

*Kerateine*—10 gm. of parent protein were freshly reduced under oxygen-free nitrogen with 4.65 gm. of thioglycolic acid at a pH just pink to phenolphthalein for 2 hours. This protein was then precipitated with trichloroacetic acid, collected on the centrifuge, washed with acetone, transferred to a mortar, and ground three times with acetone and three times with acid acetone, and dried in a vacuum desiccator.

*Metakeratin*—The parent protein, as stated above, is a mixture of kerateine and metakeratin. An attempt to convert this completely to metakeratin by oxidation with 3 per cent  $H_2O_2$  at a pH of about 8.5 led to a loss of cystine, and on acidification  $H_2S$  could be smelled. The cystine<sup>1</sup> content before oxidation of the protein was 12.27 and after 10.51 per cent.

Metakeratin without loss of sulfur may be prepared from kerateine by oxidation in mildly alkaline solution with potassium ferricyanide. 15 gm. of parent protein were suspended in 300 cc.

<sup>1</sup> The cystine content here includes the cysteine, for the protein hydrolysate is oxidized, *while still acid*, with 3 per cent  $H_2O_2$ . Then the solution is reduced with sulfite and analyzed by the Folin-Marenzi procedure.

of  $\text{H}_2\text{O}$  and  $\text{M NaOH}$  was added with constant stirring until a faint pink color developed with phenolphthalein; after some time the protein was completely dissolved. 0.2  $\text{M}$  potassium ferricyanide was added with a burette until the yellow color did not disappear instantly. The protein was dialyzed until acid to litmus; this gave an opalescent solution free of precipitate, but easily coagulated by traces of acetic acid. The protein was precipitated with dilute acetic acid, washed with acetone, and dried in a vacuum desiccator.

*Carboxymethylkeratine and Its Fractionation*—50 gm. of parent protein were suspended in 1000 cc. of water, 20 cc. of  $\text{M NaOH}$  added, and nitrogen led through the mixture. 32.5 gm. of thioglycolic acid were neutralized to phenol red and 250 cc. of 3.4  $\text{M}$  phosphate buffer, pH about 7.4, added to it; this solution was added to the protein. After 3 hours 75 gm. of recrystallized iodoacetic acid, neutralized to phenol red, were added to 250 cc. of the same buffer, and added to the protein-thioglycolate solution. After 2 hours the protein was precipitated by adding enough solid  $(\text{NH}_4)_2\text{SO}_4$  to give a 50 per cent saturated solution. The protein was filtered off, washed with 50 per cent saturated  $(\text{NH}_4)_2\text{SO}_4$ , and redissolved in 1500 cc. of  $\text{H}_2\text{O}$  + 20 cc. of  $\text{M NaOH}$ . It was again reduced with thioglycolic acid and allowed to react with iodoacetic acid exactly as in the procedure above. The protein was precipitated with  $(\text{NH}_4)_2\text{SO}_4$  at 50 per cent saturation and collected on a centrifuge, and the supernatant liquid was discarded. This protein was then dissolved in 1200 cc. of 0.1  $\text{M}$  sodium acetate and fractionated by precipitation at 35 per cent saturation with  $(\text{NH}_4)_2\text{SO}_4$ . A second fraction was obtained from the filtrate by 60 per cent saturation with  $(\text{NH}_4)_2\text{SO}_4$ . The fractions were purified by repeating this procedure several times. The first fraction, Fraction A, represents the bulk of the material; the final yield was 30 gm. of dry protein. The other fraction, Fraction B, gave a yield of only 2 gm. of dry protein.

Another preparation of carboxymethylkeratine was prepared from freshly reduced parent protein by treatment with iodoacetate at pH 9.0 to 9.5 for 40 hours, the iodoacetate being added in intervals and to a large excess, in an attempt to substitute the amino groups as well as the  $-\text{SH}$  groups. The analysis shows that no detectable substitution of the free amino groups occurred,

and that the long alkaline treatment caused a loss in sulfur, as is shown in Table I.

*$\alpha$ -Carboxyethyl-, Carbamylmethyl-, and Hydroxyethylkeratine—*These three preparations were prepared in almost identical manner 10 gm of parent protein were suspended in 100 cc. of  $H_2O$ , for each preparation, and sufficient M NaOH to give a light pink color with phenolphthalein, without attempting to dissolve the protein completely 4.65 gm. of thioglycolic acid neutralized to phenolphthalein were added to each, and the flasks stood for 3 hours under a stream of oxygen-free nitrogen. Then either 9.1 gm of  $\alpha$ -bromopropionic acid (neutralized to phenolphthalein) or 11.0 gm. of iodoacetamide, or 9.5 gm of freshly distilled iodoethyl alcohol were added. From time to time additional NaOH was added to maintain the pH at a light pink to phenolphthalein. After 2 hours the preparations made with iodoacetamide and iodoethyl alcohol were precipitated with acetic acid, washed with acetone, and then the suspensions dialyzed against distilled  $H_2O$  for 3 days The preparation with  $\alpha$ -bromopropionic acid stood overnight, was reduced in a similar manner as above, and treated a second time with bromopropionic acid for 4 hours The protein was then dialyzed for 3 days, precipitated with trichloroacetic acid, washed with acetone, and dried in a vacuum desiccator The analytical results are listed in Table I.

*Isoelectric Points—*The isoelectric points were estimated by the method of Michaelis and Rona (10) The proteins with the higher isoelectric points were dissolved in 0.1 M sodium acetate and precipitated with 0.1 M acetic acid (final concentration of acetate ion was 0.01 M), while the proteins of the isoelectric zone not covered by the pH range of acetate buffer were dissolved in 0.1 M sodium lactate and precipitated with lactic acid pH of the mixtures was determined with the glass electrode.

*Digestion—*Purified trypsin (16) and Fairchild's pepsin were used. The soluble proteins were digested with great rapidity (no precipitate with trichloroacetic acid or sulfosalicylic acid after 10 to 15 minutes at  $37^\circ$ ). Those proteins not entirely soluble at this pH were digested more slowly but at a rate comparable to other coagulated proteins.

*Methods of Analysis—*All the proteins were analyzed for nitrogen by Teorell's (17) micro-Kjeldahl method, for sulfur by the method

of Elek and Hill (18), and for cystine by the Folin and Marenzi (19) procedure, with a Zeiss-Pulfrich photometer. If the protein contained both cystine and cysteine the acid hydrolysates were oxidized with 3 per cent  $\text{H}_2\text{O}_2$ , and then the excess  $\text{H}_2\text{O}_2$  was removed by the sulfite used in the cystine reduction. Cysteine was analyzed for by the iodoacetate method of Mirsky and Anson (4). Amino nitrogen was determined by the method of Van Slyke (11) in the constant volume apparatus. The proteins were dissolved by grinding in a mortar with 10 cc. of 0.1 M NaOH and diluted to 50 cc. 5 cc. of solution containing 20 mg. of protein were used for analysis; the nitrite and protein were added prior to the addition of the acid. The time of reaction with nitrous acid was 30 minutes.

#### SUMMARY

Keratin of wool is reduced by sodium thioglycolate to kerateine. This can be oxidized to metakeratin which differs from native keratin by its amorphous character, solubility in alkali, and by its digestibility with pepsin or trypsin.

The H of the  $-\text{SH}$  group of kerateine was substituted by treatment with iodoacetate,  $\alpha$ -bromopropionate, iodoacetamide, and iodoethyl alcohol. The derived proteins thus obtained differ distinctly in their solubilities and in their isoelectric points. They are all digested by pepsin or trypsin. No detectable substitution of amino hydrogen occurred during the treatment, and under proper treatment no loss of sulfur occurred. The derivative obtained with iodoacetic acid is soluble enough to allow the attempt of a fractionation with ammonium sulfate. This led to two fractions differing widely in solubility and sulfur content.

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## THE EFFECT OF INGESTED FAT ON THE STEROL METABOLISM OF THE WHITE RAT\*

By H. C. ECKSTEIN AND CARLETON R. TREADWELL

(From the Laboratory of Biological Chemistry, Medical School, University of Michigan, Ann Arbor)

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This is a report of an investigation on the influence of ingested fats on the sterol metabolism of the white rat. No summation of the literature on sterol metabolism will be given, because the subject has been so recently reviewed by Bills (1). Only those contributions pertinent to this paper will be referred to.

The procedure adopted was to place young litter mates on vegetable diets. This was done because it is now generally accepted that the plant sterols are either not absorbed at all or only to a slight degree. The rations, which are described in Table I, were supplemented with a vitamin B concentrate (Mead Johnson), carotene, and viosterol. Soy bean meal<sup>1</sup> was chosen for the protein concentrate, because it contains considerable amounts of stigmasterol, a sterol which, according to Butenandt, Westphal, and Cobler (2), can be transformed *in vitro* into a product having many of the properties of the hormone found in the corpus luteum.

Table I shows that in one series of experiments the soy bean meal was supplemented with 4 times as much corn oil as in the other. In a second series of experiments soy bean oil was substituted for the corn oil. A record of food intakes was kept, a collection of the feces made, and changes in body weights recorded. The animals were killed at the end of experimental periods which varied from 5 to 7 weeks. The sterol contents of the livers, remaining

\* A preliminary report of this work has been published (*Proc. Soc. Exp. Biol. and Med.*, **32**, 1097 (1935))

<sup>1</sup> The soy bean flour used was purchased from the Battle Creek Food Company. Our analyses for fat and protein agreed well with those of the manufacturer. The manufacturer's analysis for carbohydrate content was accepted.

tissues, and the feces were then determined gravimetrically by means of the digitonin method (3). Those in the diets were determined similarly. All of these materials were first digested with boiling 7.5 per cent alcoholic sodium hydroxide for 2 hours. In the case of the feces so much material was still undissolved after the alkaline treatment that a subsequent digestion with boiling 10 per cent hydrochloric acid for 1 hour was added. The sterols were extracted from the fluids with ethyl ether. The ether extracts were freed from basic substances by washing with water until the aqueous extracts were no longer basic to phenolphthalein. The extracts from the acidic fecal digests were first washed with alkali and then with water. The residues obtained after removal of the ether were taken up in 95 per cent alcohol and diluted to definite volumes. Aliquots were used for the sterol analyses.

Most of the data obtained are shown in the accompanying tables. Table II shows that all of the rats grew well because the average gain per rat per day was well above 1 gm. Table II also shows that in general half of each of the litters ingested twice as much fat and half as much carbohydrate as their litter mates. The protein intakes differed only to a small extent. Table III shows that the larger amounts of liver sterols were generally found in the rats on the high fat diets. That summation also shows that the percentage of sterols in the remaining tissues of the rats on the high fat diet was as high and in some cases higher than the similar figure for their litter mates that had been on the low fat diet. These findings make it unlikely that the larger amounts of sterols referred to above are merely the result of sterol mobilizations. Table III also shows that larger amounts of sterols were invariably excreted than were ingested. This is indicative of a synthesis of sterols. It is also clear from Table III that there was generally an increased excretion following an increase in the ingestion of fat. The different effects observed between the excretion on high and low fat diets cannot be ascribed to a removal of sterols from the tissues of the rats ingesting more fat, because as has already been mentioned the amounts of sterols in the livers and remaining tissues of these animals were not only as high but often higher than those of their litter mates on the low fat diets. It is evident from combining the information shown in Tables II and III that larger amounts of sterols were deposited and excreted when large

amounts of fats and small amounts of carbohydrate were fed than when smaller amounts of fat and large amounts of carbohydrate were fed. The differences between the protein intakes of the rats in each litter were not sufficient to warrant the making of any deductions concerning the effect of protein ingestion on sterol metabolism. A conclusion that the synthesis of sterols can be increased by augmenting the fat content of the diet is at variance

TABLE I  
*Composition of Diets*

2 gm. of agar and 4 gm. of salt mixture\* were included in each of the diets.

Diet No	Soy bean meal	Starch	Corn oil	Soy bean oil
	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
1-A	49	25	20	
2-A	35	54	5	
1-B	49	25		20
2-B	35	54		5

\* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 37, 572 (1919).

TABLE II  
*Average Food Intakes per Day during Whole Experimental Period and Average Gain per Rat per Day*

Litter No	Diet No	Fat	Protein	Carbohydrate	Gain in weight per day
		<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
1	1-A	141	92	170	1.6
	2-A	68	79	336	1.3
2	1-A	97	63	117	1.6
	2-A	48	56	241	1.7
3	1-B	143	93	171	2.2
	2-B	76	89	386	2.4

with one made by Schoenheimer and Breusch (4) who fed considerable amounts of lard to mice and decided that ingested fats did not increase the sterol synthesis in such animals. Their data, however, show that the average synthesis of mice fed lard was 15 per cent higher than that of their controls. A production of sterols from fat is in line with an observation made by Lifschütz (5) who reported that an oxidized product of oleic acid gave

some of the color reactions of the sterols. Additional support is to be found in the recent work of Minovici (6) who reported that the cholesterol content of the blood of dogs increased when oleic acid was fed. Such experiments are of course not proof of sterol

TABLE III

*Sterol Contents of Livers, Remaining Tissues, Food, and Feces*

With the exception of Rats 6 to 11 inclusive (Litter 1), the experimental periods were of 5 weeks duration In this group the period was 7 weeks

Litter No	Diet No	Rat No	Liver sterols	Sterols in remaining tissues	Ingested sterols (a)	Fecal sterols (b)	(b - a)
1	1-A	4	per cent	per cent	gm	gm	gm
		5	1 00	0 20	0 958	2 409	1 451
		6	0 69	0 19	1 102	2 143	1 041
		7	0 41	0 19	0 976	2 056	1 080
		8	0 39	0 20	0 919	1 895	0 976
		8	0 34	0 21	0 982	2 298	1 316
	Average .		0 57	0 20	0 957	2 160	1 173
	2-A	2	0 42	0 20	0 320	0 778	0 458
		3	0 40	0 19	0 360	1 002	0 642
		9	0 28	0 17	0 354	1 223	0 868
		10	0 35	0 19	0 384	1 016	0 632
		11	0 42	0 19	0 416	1 283	0 867
	Average .		0 37	0 19	0 367	1 060	0 693
2	1-A	1-a	0 43	0 21	0 771	1 770	0 999
		2-a	0 72	0 19	0 714	2 491	1 720
		3-a	0 67	0 29	0 647	1 678	1 031
		5-a	0 48	0 24	0 569	1 455	0 886
		6-a	0 75	0 25	0 556	1 821	1 265
		7-a	0 57	0 22	0 714	1 490	0 703
	Average . . .		0 60	0 23	0 662	1 784	1 122
	2-A	8-a	0 35	0 21	0 223	0 565	0 342
		9-a	0 34	0 19	0 297	0 536	0 289
		11-a	0 30	0 23	0 214	0 840	0 626
		12-a	0 38	0 21	0 272	0 838	0 566
		13-a	0 46	0 20	0 282	0 852	0 570
		14-a	0 43	0 22	0 248	1 215	0 967
	Average		0 38	0 21	0 259	0 819	0 560

TABLE III—*Concluded*

Litter No	Diet No	Rat No	Liver sterols	Sterols in remaining tissues	Ingested sterols (a)	Fecal sterols (b)	(b - a)
			<i>per cent</i>	<i>per cent</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
3	1-B	1-b	0 61	0 16	0 568	1 134	0 566
		2-b	0 42	0 18	0 630	1 264	0 634
		3-b	0 79	0 19	0 614	1 400	0 786
		4-b	0 70	0 17	0 576	1 171	0 595
		Average .	0 63	0 18	0 591	1 245	0 648
	2-B	5-b	0 48	0 18	0 467	0 877	0 410
		6-b	0 57	0 18	0 515	0 873	0 358
		7-b	0 68	0 18	0 411	0 846	0 435
		8-b	0 31	0 15	0 342	0 690	0 348
		Average .	0 42	0 18	0 434	0 810	0 376

synthesis. His other experiments, however, showed that the sterol contents of dog livers increased after incubation. This increase was invariably the greatest after oleic acid feeding. In addition to this Minovici reported that certain insects produced larger amounts of sterols on media containing oleic acid than on those in which the acid was substituted by stearic acid, margaric acid, or starch. These findings indicate that the unsaturated fatty acid, oleic, contributes to a formation of sterols, while the saturated ones, stearic and margaric, do not. The iodine number of lard is lower than that of either corn or soy bean oil. If the degree of unsaturation of a fat is related to the amounts of sterols synthesized, one might expect a difference between our results and those of Schoenheimer and Breusch. Another explanation could be that the last mentioned authors used mice, while we employed rats. It is evident that more work must be done on these questions.

We are not prepared to state whether the larger amounts of sterols in the livers of the rats were due to ingested sterols, fats, or both. More work is now being done on this phase of the question.

#### SUMMARY

1. Data are presented which show that in general the amounts of sterol in the livers of growing white rats are greater when the

percentage of corn or soy bean oil in their diets is increased. These larger amounts cannot be ascribed to sterol mobilizations because the sterol contents of the remaining tissues of the rats on high fat diets were as great if not greater than the amounts found in the remaining tissues of their litter mates on low fat diets.

2 The most marked negative balances, as determined by the differences between dietary and fecal sterols, were obtained when the corn or soy bean oil contents of the diets were increased.

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## THE PHOSPHORUS METABOLISM OF INVERTEBRATE NERVE

BY GEORGE L. ENGEL AND R. W. GERARD

(*From the Marine Biological Laboratory, Woods Hole*)

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Previous work (Gerard and Wallen, 1929; Gerard and Tupikow, 1931; Tang, Tang, and Gerard, 1932) has indicated that the soluble phosphorus compounds of vertebrate and invertebrate nerve play a rôle in the important chemical sequence of activity. There are grounds (Gerard, 1932, 1934) for supposing that in nerve, as in muscle, phosphocreatine breaks down in the most intimate relation with the physiological response, energy for its resynthesis being derived next immediately from the succeeding breakdown of adenylypyrophosphate (adenosinetriphosphate). Recent experiments (Lohmann, 1934) with muscle brei suggest that in muscle this order may be reversed. In frog nerve, but not in crab, there is a burst of heat production as an early recovery phase after conduction. The present experiments started from the consideration that if this heat phase represented the adenylypyrophosphate link of the chain, then the nucleotide compound would be absent or unchangeable in crustacean nerve, though active in frog nerve metabolism (see Hill, 1934; Gerard, 1934). The results demonstrate that adenylypyrophosphate plays an intimate rôle in the metabolism of these nerves, probably quite analogous to that in frog nerve. In addition, they supply further evidence for the sequence phosphagen—adenylypyrophosphate in breakdown and resynthesis.

### *Method*

*Analytical*—Phosphates were determined by the Fiske and Subbarow (1925) method, as employed by Lohmann and Jendrassik (1926). To obtain sufficient color for reliable readings (in four fractions each of a control and two experimental portions) 0.5 to



0.8 gm. of tissue (four to seven lobsters) was used and occasionally standard P (up to 0.02 mg) added to a fraction. Cooled nerves were ground in 6 cc. of iced 10 per cent trichloroacetic acid and the suspension filtered in 10 to 15 minutes. The filtrate was neutralized with NaOH until pink to phenolphthalein, adjusted to 0.05 N HCl by addition of N HCl, and fractionated as follows. (The original 5 cc. of filtrate became 7 cc. after treatment. It is assumed that soluble P is equally distributed between filtrate and residue in calculating the results.)

*Fraction I*—A 2 cc. aliquot was analyzed immediately for reactive P. This represents essentially inorganic P.

*Fraction II*—A second aliquot of 2 cc. was hydrolyzed for 20 hours at room temperature before analysis. The increase in active P (*Fraction II*—*I*) is a measure of arginine P (Meyerhof, 1930).

*Fraction III*—Another aliquot of 1.5 cc. was brought to 1 N with HCl, hydrolyzed at 100° for 7 minutes, and the reactive P determined. The increase above *Fraction II* is taken as measuring pyrophosphate P (Lohmann, 1928); and *Fraction III* itself represents the total of the labile fractions.

*Fraction IV*—A final 1 cc. aliquot was ashed with 1.5 cc. of 10 N  $\text{H}_2\text{SO}_4$  plus a few drops of concentrated  $\text{HNO}_3$  (Teorell, 1931). The total acid-soluble P was now measured; the increase over *Fraction III* represents the stable fraction.

This fractionation is crude. *Fraction I*, though predominantly inorganic phosphate, may include some phosphorus of dihydroxyacetone phosphoric acid, which breaks down rapidly in alkaline solution. In addition, during the 15 minutes testing in 0.7 N acid and molybdate, a possible hydrolysis of some nucleotide polyphosphates and lactic acid intermediaries is not excluded. However, since no significant hydrolysis of the phosphoarginine occurs within this time, and since the color remains constant for at least 10 minutes longer, any error would be negligible.

The difference between *Fractions II* and *I* probably represents arginine phosphate fairly accurately, and the present values check very closely with those obtained by Tang, Tang, and Gerard (1932) by the Ca precipitation method<sup>1</sup>

<sup>1</sup> In an earlier series of results (1933), in which *Fraction I* was determined on the original trichloroacetic acid extract before neutralization and in which

The proportions and kinds of phosphate compounds which are hydrolyzed in Fraction III cannot be accurately stated, but in all likelihood adenylypyrophosphate (adenosinetriphosphate) is mainly involved. By the procedure no distinction can be made between adenylypyrophosphate and free pyrophosphate ion, and there would be no change if adenylypyrophosphate breaks down to adenylic acid and pyrophosphate rather than orthophosphate, as reported for muscle in some conditions (Ferdmann and Feinschmidt, 1935). Pyrophosphate will be taken to mean adenylypyrophosphate in the further treatment. Other compounds that would be partially broken down in this fraction include hexosediphosphoric acid (30 per cent hydrolyzable), hydroxyacetone phosphoric acid (50 per cent), glyceric aldehyde phosphoric acid (50 per cent), and phosphopyruvic acid (50 per cent). It is not certain that these compounds occur in invertebrate nerve, but in any event, since they serve as intermediates in lactic acid formation, their content at any time would be small, except possibly for the hexosediphosphate.

The stable fraction determined by the increase after wet ashing represents the compounds wholly resistant to 7 minutes acid hydrolysis as well as the unhydrolyzed portions of the others just considered. The stable compounds include hexosemonophosphoric acid, phosphoglyceric acid, glycerophosphoric acid, and the nucleotides minus any pyrophosphate. The acid ashing method, though criticized, gave very consistent results.

*Nerve Preparation*—Nerves of the large claw and the walking legs and the abdominal ganglion chain of the lobster (*Homarus americanus*) were utilized. The animals were placed on ice 1 to 2 hours before use, and the nerves dissected out into dry, iced mortars, stretch, as in pulling through joints, being carefully avoided. The tissue was gently blotted with filter paper, weighed

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single animals were dissected in  $\frac{1}{4}$  to  $\frac{1}{2}$  hour, values for inorganic P agreed accurately with present ones, but the arginine phosphate was twice as high. Since the 1933 data were less regular and complete and in disagreement with results by the Ca method, which had been fully controlled, we have used these only in summary fashion. All general findings were, however, entirely similar in the two sets. No indications of a creatine phosphate fraction was obtained, although this should have been obvious in hydrolysis curves. (See also Needham, Needham, Baldwin, and Yudkin (1933) )

rapidly on a torsion balance with a glass hook, and either ground at once or placed under the desired conditions. No drying occurred, water condensing rather from the moist air onto the cold tissue. When exposed at room temperature, however, nerves lost 5 per cent of their weight in 10 minutes; 12 per cent in an hour. Tissue from different animals was distributed equitably among the samples.

Nerves were exposed to oxygen or hydrogen on the walls of a 50 cc. dropping funnel closed with a stopper containing a straight tube stop-cock, and containing a few cc. of artificial sea water (NaCl, KCl,  $\text{CaCl}_2$  (Garrey, 1915) ) Gas was bubbled in through the water for 10 minutes after placing the nerves, and flushed through subsequently every hour. The temperature varied from 20–25° in different experiments

### *Results*

*Fresh Nerve*—The phosphate distribution in freshly dissected nerves is shown in Table I. The difference in different types of tissue is due in part to variable dissection time, for the same changes take place as during rest in oxygen, although slower, even when the nerves as dissected are kept at about 5° and in air. Arginine phosphate, for example, is generally higher when 1½ hours elapse between dissection of the first nerve and of the last than when this time is only 1 hour. A rough correction for changes during dissection is obtained by taking half the dissection time, correcting for temperature difference (times 0.2), and assuming changes in  $\text{O}_2$  are linear with time. A 10 per cent lower arginine P and a 10 per cent higher pyrophosphate result. However, there is a real difference in the P values for claw nerves and ganglia of the same animals (two experiments); although calculated per unit of dry weight (nerves 21 per cent solid, ganglia 24 per cent (Chang, 1931)) even this difference is within experimental error. The variation from batch to batch is inherent in the material, duplicate determinations on the same animals agreeing within 5 per cent. Earlier data showed Fractions II and III (the only ones determined) to be 10 per cent greater in spider-crab nerve than in mixed nerve and ganglia of lobster. Data on frog nerve and *Limulus* muscle are included for comparison.

*Rest in Oxygen*—In Table II are summarized the changes during

TABLE I  
Fresh Tissue

Tissue	No of animals	Dissection time	P, mg per cent of wet weight					
			Fraction I Inorganic	Fraction II - I Arginine	Fraction II Labile	Fraction III - II Pyro	Fraction IV - III Stable	Fraction IV Total
Lobster, 1934 Peripheral nerve		hrs						
	6*	2	10 4	4 3		8 7	9 0	32 4
	6**	1 2	12 4	3 6		11 9	10 3	38 2
	5	0 5	11 1	4 2		11 2	11 1	37 6
	5	0 5	11 3	5 7		6 8	10 4	34 2
	22	1	11 3 (11 4)	4 5 (4 3)	15 8	9 7 (9 9)	10 2 (10 1)	35 6 (35 7)
	7*	2	14 3	6 5		14 4†	9 6†	44 8
	7**	1 2	17 5	4 3		9 6	11 4	42 8
	14	1 5+	15 9 (16 0)	5 4 (5 0)	21 3	11 0 (11 4)	11 1 (11 2)	43 8 (44 0)
Mixed nerve and ganglia	3	1 5	12 1	4 7		5 2	15 2	37 2
	3	1	10 7	8 0		5 7	16 7	41 1
	3	1	12 8	4 5		12 1		
	3	1	14 3	7 4		10 4		
	3	2 5	9 0	9 7		11 9	8 0	38 6
	3	2	11 9	5 8		9 8	9 8	37 3
	3	2	11 4	4 3		8 6		
	3	2	9 9	5 0		5 0		
	24	1 5+	11 6 (11 8)	6 2 (5 8)	17 8	8 6 (8 8)	12 1 (12 1)	38 5 (38 5)
Lobster, 1933								
Mixed nerve and ganglia	10	0 3	11 6	13 4	25 0	10 9		
Mixed nerve	†			5 1				41
Spider-crab nerve, 1933	27	0 5			29 4	10 3		
Frog nerve	§		10	8		6 5	14	38
Limulus muscle			75	42	117	40	46	203

The values in parentheses are corrected for estimated change during the time of dissection

\* Tissues obtained from the same animals

\*\* Tissues obtained from the same animals

† Values questionable, given half weight

‡ Data from Tang, Tang, and Gerard (1932)

§ Data from Gerard and Tupikow (unpublished)

|| Data from Engel and Chao (1935)

TABLE II  
*Rest in Oxygen*

Condition	Tissue*	P, mg per cent			
		Inorganic	Arginine	Pyro	Stable
Initial values	Lobster, 1934				
	Ganglion (14)	15.9	5.4	11.0	11.1
	Nerve (22)	11.3	4.5	9.7	10.2
	Mixed (12)	12.5	6.1	8.5	12.0
Changes during 1 hr in O <sub>2</sub>	Lobster, 1933 (10)	11.9	13.4	11.3	
	Crab, 1933 (4)		31.0	9.2	
	Lobster, 1934				
	Ganglion	-0.5	+2.2(2.6)	-3.1(3.5)	-0.3(0.4)
Changes during total time in O <sub>2</sub> (8 hrs, 1934; 5 hrs, 1933)	Nerve	-0.3	+1.8(2.0)	-1.8(2.0)	+0.3(0.4)
	Mixed	-0.6	+0.9(1.3)	-0.6(0.8)	-0.1(0.1)
	Lobster, 1933	+1.1	+3.3	-1.7	
	Crab, 1933		+3.9	-3.2	
Changes during 1st hr	Lobster, 1934				
	Ganglion	+0.4	+0.9	-1.3	-0.7
	Nerve	+1.4	+1.6	-1.0	-2.7
	Mixed	+2.0	-0.7	+2.7	-3.4
Changes in O <sub>2</sub> after 1st hr	Lobster, 1933	+3.6	+3.8	+0.9	
	Crab, 1933		+1.4	-2.2	
	Lobster, 1934				
	Ganglion	+0.9	-1.3	+1.8	-0.4
Changes in O <sub>2</sub> after 1st hr	Nerve	+1.7	-0.2	+0.8	-3.0
	Mixed	+2.6	-1.6	+3.3	-3.3
	Lobster, 1933	+2.5	+0.5	+1.2	
	Crab, 1933		-2.5	+1.0	
					Total
					43.8
					35.7
					39.1
					-1.7
					0.0
					-0.4
					-0.5
					-0.7
					+0.6
					+1.2
					-0.7
					+1.0

\* The values for tissue given in parentheses represent the number of animals averaged

† The values for P given in parentheses are corrected for estimated change during dissection

rest in oxygen, following the unavoidable stimulation of dissection. All data are obtained by direct difference between companion samples. Since similar changes occur in the control nerves during the dissection period, the total change after dissection is greater than shown (see estimated correction). The data for ten experiments, with forty-eight animals, are averaged for nerve, ganglion chain, and both.

During short rest in oxygen ( $\frac{3}{4}$  to 1 hour plus 1 to  $1\frac{3}{4}$  hours dissection) there is a slight decrease (5 per cent) in inorganic phosphate and possibly in the total phosphate, with no change in the stable fraction. More striking is a regular and distinct increase (by one-third) of arginine phosphate which is balanced by a numerically equal loss of pyrophosphate (one-fourth of the total). In four experiments with rapid dissection and short rest in oxygen this balance was exact, as it was also in the average for all experiments with peripheral nerve (arginine phosphate, +1.8 mg. per cent, pyrophosphate, -1.8). The averages for ganglion (+2.2 and -3.1) and for mixed nerve and ganglion (+0.9 and -0.6) showed less perfect agreement, but in these experiments longer dissection and exposure times were involved, and one ganglion experiment was unreliable. Earlier results on crab and lobster were less regular but in agreement.

During further rest in oxygen (8 hours) these changes are partly reversed. Inorganic phosphate increases definitely (15 per cent); total phosphate, possibly; and the stable fraction decreases in all cases (20 per cent average). Arginine phosphate again decreases somewhat (15 per cent), while the pyrophosphate lost during the 1st hour is restored. The final balance after a long period in oxygen shows, therefore, no change from the initial values for total phosphate or pyrophosphate and a dubious increase of arginine phosphate, while a definite shift of phosphate from the stable to the inorganic fraction has occurred. After only 1 hour, conversely, the only clear change is from pyrophosphate to arginine phosphate and this is subsequently reversed.

*Rest in Hydrogen*—Anoxia leads to marked and rapid changes which have reached completion or equilibrium in 5 hours (Table III). Arginine phosphate is completely lost in nerve, three-fifths in mixed ganglion and nerve; and three-fourths or more of the pyrophosphate of nerve, somewhat less with ganglia present,

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breaks down. These changes are complete by 2 hours. Inorganic phosphate increases for 5 hours, when it has doubled, at the expense of the above fractions and also of the acid-stable one which decreases two-fifths in 5 hours.

In all cases the total acid-soluble P showed an unexpected decrease, greater after 5 hours than after 2, and contrary to earlier

TABLE III  
*Anoxia*

Condition	Tissue*	P, mg per cent				
		Inor- ganic	Argi- nine	Pyro	Stable	Total
Initial values	Lobster, 1934					
	Nerve(20)	13†	6†	10†	15.4	44.3
	Mixed(12)	10.5	6.2	8.8	8.9	34.4
	Lobster, 1933(5)	12.6	14	13.9		
	Crab, 1933(3)	31.4		10.5		
Change during 2 hrs in H <sub>2</sub>	Lobster, 1934					
	Nerve	+10.5	-5.8	-6.8	-2.8	-5.0
	Mixed	+6.6	-3.3	-5.5	+0.7†	-1.5
	Lobster, 1933	+12.8	-4.7	-7.8		
	Crab, 1933	+12.1		-8.6		
Change during 5-9 hrs in H <sub>2</sub>	Lobster, 1934					
	Nerve				-6.6	-6.8
	Mixed	+10.6	-3.4	-5.6	-0.6†	-1.2†
Change in H <sub>2</sub> after first 2 hrs	Lobster, 1934					
	Nerve				-3.8	-1.8
	Mixed	+4.0	-0.1	-0.1	-1.3†	+0.3†

\* The values in parentheses represent the number of animals averaged

† Values estimated for this series

‡ Values on mixed tissue are not all from complete parallel experiments, therefore not in full agreement. Those marked are least certain. Probably the stable fraction change at 5 hours should be considerably greater.

findings on the frog and crustacean nerve (Gerard and Wallen, 1929; Tang, Tang, and Gerard, 1932). It is unlikely that a synthesis of insoluble compounds occurs under these conditions, and the previous results indicated the reverse change. More probably, anoxia enhanced a loss of inorganic phosphate into fluid

TABLE IV  
*Recovery from Anoxia*

Condition	Tissue*	P, mg per cent†			
		Inorganic	Arginine	Pyro	Stable
Values after 3 hrs in H <sub>2</sub>	Lobster nerve, 1934(14) Crab, 1933(3)	23 5(+10 5) 33 7(+7 5)	0 2(-5 8)	3 2(-6 8) 3 5(-6 8)	13 4(-2 6) 40 3(-4 7)
Change during 1 hr in O <sub>2</sub>	Lobster nerve, 1934	-4 5	+2 5	+2 8	-2 7
Change during 4-6 hrs in O <sub>2</sub>	Lobster nerve, 1934 Crab, 1933	-7.4(+3 1) -1 9(+5 6)	+4 2(-1 6)	+3 3(-3 5) +2 6(-3 8)	-4 6(-7 2) -4 5(-9 2)

\* The values for tissue given in parentheses represent the number of animals averaged.

† The values for P given in parentheses give the change from the initial values.



condensed or sprayed onto the tissue, and which unfortunately was not analyzed. In oxygen-lack the diffusible fractions are increased, and nerve permeability may also be greater (Hill, 1934; Gerard, 1934). Eggleton (1933) has observed an out-diffusion of phosphate when its concentration in muscle cells is increased relative to that in the interspaces.

*Rest in Oxygen following Hydrogen*—Nerves (no ganglia were used) placed in oxygen after a period of anoxia show a marked recovery (Table IV). Arginine phosphate regenerates strongly and returns from zero to the level for fresh nerve in 6 hours. Pyrophosphate is restored from less than one-third of the original concentration to two-thirds after an hour in oxygen, but shows little further increase in another 5 hours. Inorganic phosphate falls from twice the initial value to 40 per cent more. The stable fraction, however, decreases further, to half the initial value, and the total soluble phosphate continues to fall from the value at the end of anoxia by an amount equal to the loss of the stable fraction.

*Stimulation*—In the 1933 series, in eleven experiments on lobster and crab, stimulation in oxygen with 10 to 15 shocks a second<sup>2</sup> caused a loss of 1.2 mg per cent of arginine phosphate and 0.5 of pyrophosphate, compared to the resting controls. Since, on the one hand, stimulation was probably far from the maximal and, on the other, local electrode effects were not excluded, these results are only suggestive.

#### DISCUSSION

The breakdown of adenylypyrophosphate in conjunction with a synthesis of phosphoarginine is especially interesting. Since some injury and excitation during dissection are unavoidable, the subsequent changes may be considered as a recovery process. This is further evidenced by the early synthesis of arginine phosphate during rest in oxygen. The results strongly indicate that the following reaction occurs during aerobic recovery of invertebrate nerve.

Adenylypyrophosphate + 2 arginine  $\rightarrow$  2 arginine phosphate + adenylic acid  
In at least half the experiments, those of short duration, the balance was quantitatively exact. When longer times were used,

<sup>2</sup> We are indebted to Dr. H. Davis for the loan of a neon stimulator for these experiments.

the arginine phosphate synthesis was greater than the apparent adenylypyrophosphate breakdown. This is undoubtedly the result of the observed resynthesis of adenylypyrophosphate at a later stage, after arginine phosphate is rebuilt. The main reaction in question is probably complete in 1 hour.

Lohmann (1934) concluded from experiments on muscle brei that the reverse reaction is associated with muscle activity.

- (1) Adenylypyrophosphate  $\rightarrow$  adenylic acid + 2 phosphoric acid
- (2) Adenylic acid + 2 creatinephosphoric acid  $\rightarrow$  adenylypyrophosphate + 2 creatine

Any involvement of pyrophosphate has been challenged by Lundsgaard (1934), studying intact iodoacetate muscle, and in fact Meyerhof and Lohmann (1931) earlier observed that muscle extracts did not form phosphagen unless adenylypyrophosphate was present and that the nucleotide broke down as creatine phosphate formed. More recently Needham and van Heyningen (1935) have been able to obtain this reaction in either direction in muscle brei.<sup>3</sup>

On aerobic recovery following anoxia all the arginine phosphate is reformed in 5 hours, while two-thirds of the adenylypyrophosphate is restored in 1 hour with no further significant increase. The stable fraction decreases by one-third during this period, probably with the formation of some compounds that appear partially in the pyrophosphate fraction. Presumably a balance is established in an hour between adenylypyrophosphate breakdown and its resynthesis from other phosphate sources.

A loss of 4.2 mg. per cent of adenylypyrophosphate P would be required for the resynthesis of arginine phosphate, actually a gain of 3.3 mg. per cent is observed, so that a total synthesis of 7.5 mg. per cent of P as nucleotide is required. The phosphate for this could come from the stable fraction (maximum possible, 4.6 mg. per cent) and the inorganic one. In muscle brei, adenylic acid can be phosphorylated by phosphocreatine and by phosphopyruvic acid, probably acting via the phosphagen (Parnas, Ostern, and Mann, 1935; Needham and van Heyningen, 1935). The present evidence is against arginine serving as a donor in nerve. (This could

<sup>3</sup> An excellent statement of the case for muscle, indicating this reversibility and its functional significance, has been published by Parnas and Mann (1935).

possibly occur during the later stages of rest in oxygen.) Some of the lactic acid intermediates in the stable fraction are probably involved, although they cannot be the sole source of phosphate.

The extensive breakdown of both arginine and pyrophosphates during continued anoxia is in accord with previous results on frog and lobster nerve (see Gerard and Tupikow, 1931). To maintain even the resting nerve in its physiological state of responsiveness requires a continuous supply of energy (Gerard, 1932), derived ultimately from the oxidation of foodstuffs. During anoxia the necessary energy may be partly released by hydrolysis of the phosphorus compounds, which are later restored, when oxygen is again available, with the aid of oxidative energy. The cycle of breakdown and resynthesis presumably proceeds uninterruptedly under normal conditions and in any case there is a definite correlation between oxidation and phosphorylation (See also Runnstrom, Lennerstand, and Borei (1934), Runnstrom and Michaelis (1935), and Lipmann (1934). Contrast the view of Sacks and Sacks (1935), that, in muscle, acidity controls phosphorylation.)

Besides anoxia, long standing in oxygen leads to hydrolysis of arginine phosphate. Whether this results from depletion of substrate and reduced oxidative energy, or is a consequence of bacterial action, cannot be stated.  $\text{CO}_2$ , easily formed by bacteria, leads to phosphagen breakdown, even in 5 per cent gas concentration (Gerard (1932) p. 499) and bacterial effects may be manifest in dogfish (Root, 1934) or mammalian (Schaffer, Chang, and Gerard, 1935) nerve in 7 hours or less, although mainly at higher temperatures.

The stable fraction tends to decrease, irreversibly, under all conditions studied except short rest in oxygen. It diminishes more rapidly under anoxic conditions than during prolonged aerobic rest, and after anoxia continues to fall even in oxygen. If this represents a loss of phosphorylated carbohydrate intermediates, these are presumably being destroyed more rapidly than they are reformed from glycogen, etc., of which a plentiful store exists (Holmes, 1929). Pending more specific measurement, this does not merit further discussion.

The total soluble phosphate remained unchanged in oxygen but diminished in oxygen-lack and continued to fall when oxygen was again admitted. The anoxic loss is most probably due to diffu-

sional losses, as discussed, and the subsequent loss in oxygen may be similar. In the latter case, however, a synthesis of insoluble phosphates may have occurred, and it is interesting that the loss of total phosphate is equal to the loss in the stable fraction.

#### SUMMARY

Inorganic P, arginine P, adenylypyrophosphate P, stable P, and total acid-soluble P fractions have been determined in lobster claw nerves and ganglia and crab nerves.

During rest in oxygen, the phosphoarginine decomposed during dissection (and attendant stimulation and injury) is rebuilt, apparently by direct reaction with adenylypyrophosphate, which decomposes. This, in turn, is subsequently resynthesized, the lactic acid intermediates probably serving in part as phosphate donors. During anoxia there is an extensive hydrolysis of both phosphoarginine (up to 100 per cent) and adenylypyrophosphate, with an attendant rise in the inorganic fraction. Subsequent exposure to oxygen results in full resynthesis of arginine phosphate in 5 hours; and of two-thirds of the adenylypyrophosphate within an hour, with no further change. Both substances are probably decreased by stimulation.

The stable fraction (hexosephosphate, etc.) regularly shows a loss of bound phosphorus after several hours, greater during or following the absence of oxygen. There is no evidence that the phosphorus of any acid-insoluble compounds (phosphoproteins, phospholipids, nucleoproteins, etc.) is involved in the aerobic metabolism of these nerves.

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## A SPECIFIC POLYSACCHARIDE FROM THE BACILLUS CALMETTE-GUÉRIN (BCG)\*

BY ERWIN CHARGAFF AND WERNER SCHAEFER

(From the Laboratories of the Pasteur Institute, Paris, France, and of the  
Mount Sinai Hospital, New York)

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During the last few years the chemical composition of a number of strains of acid-fast bacilli has been investigated in various laboratories (1). Among these strains, the bacillus Calmette-Guérin (BCG) deserves special interest because of its extensive practical application. A study of the chemistry and serology of this avirulent microorganism may ultimately bring about a better understanding of the chemical aspect of the virulence problem.

We have previously studied the chemical (2) and immunological (3) properties of the various lipid fractions of the BCG (fat, phosphatide, wax). In the present paper we report on specific polysaccharides obtained by extraction of the defatted BCG and also on some observations on the proteins of the BCG.

*Polysaccharides*—The polysaccharides were obtained from the defatted bacilli by means of acetate buffer or dilute acetic acid, similar to the methods used by Heidelberger and Menzel (4). Three separate preparations, made from as many individual batches of bacilli, yielded concordant results. The extracted material was purified by precipitation with alcohol from acid or alkaline solution. The use of alkali could not be avoided because of the peculiar behavior of certain fractions.

Two main fractions were obtained which differed considerably in their solubility. One of the compounds, designated as A frac-

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tion, is readily soluble in water and alkali. Iodine-potassium iodide solution produces a blue-violet coloration similar to that obtained with an amyloextrin. The other substance, designated as S fraction, is almost insoluble in water and alkali and requires rather strong acids for solution. The substance is precipitated from its solutions by alkali or alcohol without appreciable change. This fraction consists of about equal parts of inorganic material and carbohydrates. A microchemical examination, for which we are indebted to Dr. A. Benedetti-Pichler of New York University, Washington Square College, revealed the inorganic constituents of the S fraction remaining after combustion to consist of almost pure  $\text{Ca}_3(\text{PO}_4)_2$ . We deal here apparently either with the calcium salt of a phosphorylated polysaccharide or with an extremely strong adsorption compound of a polysaccharide with calcium phosphate, which is dissociated only with the utmost difficulty. By treatment with ammonia, part of a polysaccharide is split off, which, however, does not seem to be identical with the A fraction.

The quantities of bacilli used and the yields of polysaccharides are given in Table I.

From the combined A fractions, after further purification, a polysaccharide was obtained, which was entirely free of proteins, dextrorotatory,  $[\alpha]_D = +77.4^\circ$ , and did not reduce Fehling's solution. About 77 per cent of its weight was obtained as reducing sugars upon acid hydrolysis. Aldopentoses and aldohexoses, but no ketoses were present. This polysaccharide was a weak acid with an acid equivalent of 1244. It contained 0.8 per cent nitrogen, 0.9 per cent phosphorus, and 2.9 per cent of amino sugars<sup>1</sup> (calculated as glucosamine). If the latter really form part of the polysaccharide molecule, a minimum molecular weight of about 5000 can be assumed.

Only 296 mg. of the polysaccharide were available for the study of the products formed by acid hydrolysis. These consisted mainly of two sugars: 46 per cent of the polysaccharide were isolated as *d*-mannose; 8 per cent as *d*-arabinose. Together with the amino sugar mentioned before about three-quarters of the reducing sugars contained in the polysaccharide could thus be accounted for.

<sup>1</sup> The quantitative estimation of the amino sugars was kindly carried out by Dr. K. Meyer of Columbia University, College of Physicians and Surgeons, according to his method (5).

Neither galactose nor uronic acids were present in our preparation. Under the conditions of hydrolysis used no compounds whatsoever with acid function were observed.

The non-reducing fraction of the polysaccharide apparently contains mositol, the presence of which was demonstrated by color reactions, but due to the minute amount present no crystals were obtained.

Both the A and the S fractions showed specific precipitation with anti-BCG horse sera up to a dilution of 1:1,000,000. By intravenous injection of the A fraction no antipolysaccharide serum was produced in rabbits. Further experiments will be necessary to decide whether it is possible to isolate true antigens instead

TABLE I  
*Polysaccharides Isolated from BCG*

Experiment No	Weight of dry bacteria	Solvent used for extraction	A fraction		S fraction	
			Weight	Per cent of dry bacteria	Weight	Per cent of dry bacteria
1	gm 60 0	Acetate buffer, pH 4.1	gm 0 17	0 28	gm 0 16	0 27
2	110 0	1 N acetic acid	0 32	0 29	0 28	0 26
3	90 0	1 " " "	0 37*	0 41*	0 45*	0 50*

\* These fractions were not as highly purified as those in Experiments 1 and 2.

of the haptens obtained by us, in accordance with the findings on Type I pneumococcus polysaccharides (6). Our polysaccharides apparently are species-specific, as cross reactions were observed with antitubercle bacilli sera.

Another polysaccharide which accompanies the fat and phosphatide of the BCG (2), analogous to the A-8 fractions studied by Anderson and collaborators (7), was serologically entirely inactive.

*Proteins*—For the isolation of BCG proteins use was made of the method developed by Heidelberger and Kendall (8). In the case of the BCG it was not possible to extract a nucleoprotein from the defatted bacteria at pH 6.2. Also the protein fractions extracted at pH 7.7 and 9.2 were so minute that a study of their



properties could not be made. Only the subsequent treatment of the bacilli with 0.5 per cent potassium hydroxide yielded a larger amount of protein (1.6 per cent of the dry bacteria). This substance, which in certain regards resembles the "alkali-soluble" protein from human tubercle bacilli of Coghill (9) and the K fractions of Heidelberger and Menzel (10), was a true antigen. It reacted in the complement fixation test with antiprotein sera and with antisera against BCG and bovine tubercle bacilli up to a dilution of 1:10,000. The protein did not react with the anti-BCG

TABLE II  
*Composition of BCG*

Fraction	Per cent of dry bacteria	Serological activity in dilution	Smallest amount of antigen demonstrable	No of bacterial cells furnishing minimum amount of antigen demonstrable
			$\mu\text{gm}$	$10^6$
Fat . . . . .	9.7			
Wax . . . . .	11.1			
Phosphatide	4.6	1:1,000,000	0.3	65
Polysaccharide accompanying lipid fractions . . . . .	0.5			
Specific polysaccharides*	0.5	1:1,000,000	0.25	500
" protein	1.6	1:10,000	30	18,750
Residue . . . . .	72.0			

\* A and S fractions

phosphatide serum (3), a fact which is important with regard to the specificity of the BCG phosphatide.

Summarizing the results of our studies of the chemical constituents of the BCG, we find that for each of the three groups which are the major cell constituents a representative antigen has been isolated, namely a lipid, a polysaccharide, and a protein. The distribution of the isolated fractions, together with data on their antigen activity, is given in Table II. We also attempted to compute the number of bacteria which are necessary to furnish the minimum amount of antigen still demonstrable. This was based on the supposition that  $10^{10}$  cells are contained in 1 mg. of bacteria (11). This calculation may not be devoid of interest,

since in chemical work with bacteria one has to consider as the basic biological unit the bacterial cell. Each chemical compound that is isolated from bacteria must be present in a concentration of at least 1 molecule per cell. As the number of molecules per single cell can be estimated, a limiting value may be computed below which the concentration of any genuine cell constituent may not drop.

#### EXPERIMENTAL

##### *Preparation of Bacteria*

The cultures used for all experiments were 6 to 8 weeks old and were obtained by cultivating the BCG on the Sauton medium (12) at 38°. The BCG was a culture supplied to us by Dr. Guérin of the Pasteur Institute, Paris. Two different methods were employed for defatting and extracting the bacteria. In Experiment 1 (see Table I) the bacilli were first filtered on a large folded filter. They were then transferred to a large Buchner funnel and washed with distilled water in the presence of thymol. The well drained bacilli were suspended in 350 cc. of alcohol cooled to -10°. After the mixture was shaken for 20 minutes, the bacilli were filtered off, washed with alcohol-ether, ether, and finally with absolute ether, and dried *in vacuo* over P<sub>2</sub>O<sub>5</sub>. The dry bacilli were shaken again with 500 cc of alcohol-ether for 45 minutes, washed as above, and dried *in vacuo*. The bacilli then were pulverized to a very fine yellow powder and dried to constant weight.<sup>2</sup> In Experiments 2 and 3 (see Table I) the bacilli were freed of the fat and phosphatide according to the method of Anderson (13).

##### *Isolation of Polysaccharides*

For the extraction of polysaccharides the bacterial powder was treated with 0.2 N acetate buffer at pH 4.1 (4 parts of acetic acid, 1 part of sodium acetate) or with 1 N acetic acid containing 0.5 per cent of phenol. We describe here Experiment 2 only. The finely ground bacteria, 110 gm., were digested with an equal volume of 1 N acetic acid. After a short period enough acetic acid was added to bring the volume of the extraction fluid to 500 cc. The

<sup>2</sup> In order to exclude inhalation of the bacterial powder, all operations were carried out in specially constructed glass cases where the material could be handled without danger.

mixture was kept 3 days in the ice box under frequent shaking and then centrifuged. The extraction of the bacteria was repeated five times under the same conditions. Each time the serological activity of the extract was controlled by means of the precipitation test with anti-BCG horse serum. The solution obtained from the last extraction, even undiluted, showed only feeble precipitation. The combined extracts (about 3 liters) were concentrated *in vacuo* to 50 cc. To the remaining brownish turbid solution 240 cc. of absolute alcohol were added. After 3 days in the ice box the precipitate was filtered and repeatedly washed with alcohol and ether.

The precipitate was taken up in 30 cc. of water and 1 cc. of glacial acetic acid. A considerable amount of undissolved material was centrifuged off and washed with warm water. From this insoluble residue 43 mg. of a protein were extracted by 2 N potassium hydroxide, which was serologically almost inactive. The material not dissolved by potassium hydroxide yielded, on treatment with 40 per cent acetic acid and reprecipitation with alcohol, 173 mg. of a white powder that was essentially identical with the S fraction to be described later. From the clear supernatant liquid (about 47 cc.) the water-soluble polysaccharide was precipitated by the addition of 200 cc. of alcohol. Only a part of this precipitate went into solution when taken up in a small volume of water. It was found that it contained a fraction readily soluble on the acid, and insoluble on the alkaline side. Therefore, the entire material was treated with 10 cc. of water that had been made alkaline by the addition of a few drops of potassium hydroxide solution. After centrifugation of the mixture, 1 cc. of glacial acetic acid and 70 cc. of alcohol were added to the clear supernatant solution. The water-soluble polysaccharide was precipitated in the form of a syrup which adhered to the bottom of the flask and solidified on treatment with absolute alcohol. After four more precipitations from acidified water with alcohol, the polysaccharide, designated as *A fraction*, was obtained as a fine sand-colored powder which weighed 320 mg. It was easily soluble in water. A minute quantity, when brought into contact with an iodine-potassium iodide solution, produced a blue-violet color. The reactions with orcinol and phloroglucinol for pentoses were positive; the reaction with resorcinol for ketoses as well as the Millon, biuret, and trichloroacetic acid reactions for the presence of proteins was negative.

The substance reduced Fehling's solution only after acid hydrolysis. It contained 1.1 per cent of phosphorus, 0.9 per cent of nitrogen, and 2.6 per cent of ash.

*Phosphorus (Pregl-Lieb)*—18.475 mg. gave 13.990 mg. of  $(\text{NH}_4)_3\text{PO}_4 \cdot 12\text{MoO}_3$ ; found, P 1.10.

*Nitrogen (Kjeldahl)*—12.855 mg. used 0.863 cc of 0.01 N  $\text{H}_2\text{SO}_4$ ; found, N 0.94

After a sample of the polysaccharide had been hydrolyzed with 1 N hydrochloric acid for 2 hours, it contained 76.5 per cent of reducing sugars (calculated as glucose) according to the Hagedorn-Jensen method. By the Shaffer-Hartmann method considerably lower values were obtained. This is in agreement with the findings of Munday and Seibert (14).

The second polysaccharide fraction mentioned above, insoluble in alkali, was treated with 5 cc. of 40 per cent acetic acid. The solution was centrifuged and the residue washed with 2 cc. of acetic acid of the same strength. From the combined solutions the substance was precipitated by the addition of 40 cc. of alcohol. The precipitation was repeated four times, whereupon 283 mg. of a white brittle mass were obtained, which on grinding formed a very light powder. This substance, designated as *S fraction*, was insoluble in water and alkali, and soluble in acids. When a dilute potassium hydroxide solution is slowly added to an acid solution of the substance, flocculation sets in at the turning point of bromothymol blue. No coloration was observed with iodine-potassium iodide solution. The reactions for pentoses were positive, the reactions for ketoses and proteins negative. Fehling's solution was reduced only after treatment of the substance with dilute acids. A sample, hydrolyzed with 1 N hydrochloric acid for 2 hours, was found to contain 46.7 per cent reducing sugars (calculated as glucose) according to the method of Hagedorn and Jensen. The *S fraction* contained 9.8 per cent of phosphorus and 47.9 per cent of ash. No nitrogen was found. It should be noted that the *S fraction* had practically the same composition after three as after five precipitations.

*S Fraction after Three Precipitations. Phosphorus (Pregl-Lieb)*—12.440 mg gave 88.419 mg of  $(\text{NH}_4)_3\text{PO}_4 \cdot 12\text{MoO}_3$ ; found, P 10.32

*S Fraction after Five Precipitations. Phosphorus*—15.460 mg gave 103.970 mg. of  $(\text{NH}_4)_3\text{PO}_4 \cdot 12\text{MoO}_3$ ; found, P 9.77.

*Composition of the A Fraction*

The A fractions from three batches of bacilli were combined and subjected to five reprecipitations with alcohol from their acidified aqueous solutions as described above. The combined amount of 623 mg. yielded 410 mg. of a faintly yellow powder, the mother liquors an additional amount of 16 mg. This purified polysaccharide did not differ essentially from the A fraction. It formed a weak and dextrorotatory acid and contained 77.2 per cent reducing sugars (calculated as glucose) including 2.9 per cent of amino sugars (calculated as glucosamine). Thus, 28 per cent of the nitrogen contained in the polysaccharide is accounted for as amino sugar nitrogen. The naphthoresorcinol reaction for glucuronic acid of Tollens and Neuberg, as described by van der Haar (15), was negative.

*Acid Equivalent*<sup>2</sup>—4.633, 4.904 mg. used 0.369, 0.398 cc. of 0.01 N KOH; found, acid equivalent 1255, 1232.

*Optical Rotation*—17.77 mg., dissolved in 5 cc. of water and examined in a 2 dm. tube, had  $\alpha = +0.55^\circ$ ; found,  $[\alpha]_D^{25} = +77.4^\circ$ .

*Elementary Composition*—3.850 mg. gave 6.010 mg. of CO<sub>2</sub>, 2.410 mg. of H<sub>2</sub>O; found, C 42.57, H 7.00.

5.570 mg. used 0.229 cc. of 1/70 N HCl; found, N 0.82.

15.325 mg. gave 9.074 mg. of (NH<sub>4</sub>)<sub>2</sub>PO<sub>4</sub> · 12MoO<sub>3</sub>; found, P 0.86

Of this polysaccharide 296 mg. were hydrolyzed with 25 cc. of 1 N sulfuric acid for 2½ hours. After accurate neutralization with barium hydroxide solution and centrifugation, the supernatant liquid was concentrated *in vacuo* to a volume of 4 cc. To the filtered solution 300 mg. of freshly distilled phenylhydrazine were added. The mixture, from which platelets crystallized after a short time, was kept in the cold for 24 hours. The crystals were filtered off, washed with ice-cold water, and dried; they weighed 202 mg., corresponding to 135 mg. of mannose or 45.6 per cent of the polysaccharide. After three recrystallizations from 60 per cent alcohol, 150 mg. of almost white platelets were obtained, which melted under decomposition at 194–195° (corrected). A mixture of the hydrazone with mannose phenylhydrazone showed no depression of the melting point.

<sup>2</sup> All analyses are calculated on an ash-free basis.

*Nitrogen*—5 350 mg. gave 0.499 cc. of  $N_2$  (24°, 755 mm.); found, N 10.65. Calculated for  $C_{12}H_{18}O_5N_2$  (270.1), N 10 37.

The filtrate from the mannose phenylhydrazone was treated with 0.5 cc. of benzaldehyde. The benzalphenylhydrazone was filtered off and the filtrate extracted with ether several times and concentrated *in vacuo* to a syrup. The residue was taken up in 2 cc. of 75 per cent alcohol and 300 mg. of freshly prepared  $\alpha$ -benzylphenylhydrazine (boiling point 170° at 7 mm. of Hg) in 0.75 cc. of 75 per cent alcohol were added. After 1 day in the refrigerator crystals had separated which were filtered off, washed with cooled alcohol, and dried. They weighed 50 mg., corresponding to 23 mg. of arabinose or 7.7 per cent of the polysaccharide. After three crystallizations, from 1 cc. of 75 per cent alcohol each time, 11 mg. of prismatic crystals were obtained, which melted at 175–176° (corrected) There was no depression of the melting point on admixture of *d*-arabinose  $\alpha$ -benzylphenylhydrazone prepared from the polysaccharide of tubercle bacilli wax.<sup>4</sup> A mixture of the hydrazone with *l*-arabinose  $\alpha$ -benzylphenylhydrazone showed a depression of the melting point of 9°.

*Nitrogen*—4.933 mg gave 0 381 cc. of  $N_2$  (21°, 757 mm ); found, N 8.93. Calculated for  $C_{18}H_{22}O_4N_2$  (330 2), N 8.48

The filtrate from the arabinose benzylphenylhydrazone was treated with 0.5 cc. of 40 per cent formaldehyde according to the method of Ruff and Ollendorff (16) and evaporated *in vacuo* to dryness. The residue was taken up in 0 1 cc. of water and 190 mg. of freshly prepared *o*-tolylhydrazine (boiling point 130° at 9 mm. of Hg) in 3 5 cc. of absolute alcohol were added. No trace of galactose hydrazone was obtained, even after seeding with a crystal of *d*-galactose *o*-tolylhydrazone

The mixture was treated again with 40 per cent formaldehyde and concentrated *in vacuo* to dryness. The residue was dissolved in water, filtered, evaporated, and dried. A brownish syrup was obtained which weighed 115 mg. It was treated with 96 per cent alcohol until a small amount of a solid substance remained, which formed 16 mg. of an almost white amorphous powder. This sub-

<sup>4</sup> We wish to thank Professor R. J. Anderson of Yale University who kindly supplied a specimen of this compound

stance was refluxed with 0.9 cc of hydrochloric acid (1 part of concentrated HCl in 1 part of water); the dark colored solution was heated with charcoal (norit), filtered, and concentrated *in vacuo* to 0.3 cc. No definite inositol crystals were obtained, but the solution gave a strong Scherer reaction, when tested following the method of Salkowski (17).

The solution containing the alcohol-soluble parts of the syrup mentioned above was evaporated *in vacuo*, taken up in a few cc. of water, and treated with solid barium carbonate. The mixture was evaporated to dryness on the water bath. The residue was extracted with boiling 90 per cent alcohol several times, which removed the sugars present. The undissolved barium salts should have contained all substances of acid nature. They were extracted with boiling water and dilute sulfuric acid was added to the filtered solution. The amount of barium sulfate formed (1.51 mg.) corresponds to 2.5 mg. of glucuronic acid. The reaction for uronic acids in the filtrate from the barium sulfate was negative.

#### *Composition of the S Fraction*

Of the combined S fractions, 600 mg. were dissolved in 8 cc. of 40 per cent acetic acid and the solution was clarified by centrifugation. The supernatant liquid was carefully neutralized against phenolphthalein with 10 per cent ammonia which was slowly added from a burette. The voluminous precipitate was centrifuged off, washed with dilute ammonia, alcohol, and ether, and dried. It formed 241 mg. of a white powder which contained 69.2 per cent of inorganic constituents. By a microanalysis these were found to consist of approximately equivalent parts of calcium and phosphorus; thus the presence of calcium phosphate may be deduced. The spot test for magnesium with *p*-nitrobenzene azoresorcinol according to Feigl (18) was negative. After hydrolysis of a sample for 2 hours with 1 *N* hydrochloric acid 25.4 per cent of reducing sugars (calculated as glucose) was found.

This experiment showed that part of the polysaccharide could be freed from the calcium phosphate complex. To the ammoniacal supernatant liquid (about 52 cc.) from the above precipitation, 1 cc. of glacial acetic acid and 450 cc. of 95 per cent alcohol were added. A polysaccharide was precipitated, which after treatment with absolute alcohol formed 162 mg. of a faintly

yellow powder. Since it contained 93.6 per cent of reducing sugars, as compared with 77 per cent in the A fraction, it appears probable that the polysaccharides present in the A and S fractions are not identical.

#### *Immunological Properties of Polysaccharides*

All A and S fractions examined by us were haptens, but did not exert any antigen activity. Two rabbits received nine intravenous injections of a 0.1 per cent solution of the A fraction in the course of 27 days. A total amount of 45 mg of polysaccharide was injected into each animal. The sera, which were collected 6 days after the last injection, did not contain any antibodies and did not react with the polysaccharide or with the BCG phosphatide.

All preparations gave very marked precipitation with two anti-BCG horse sera. The final dilutions varied for different preparations between 1:250,000 and 1:1,000,000. The A fraction also reacted with an antitubercle bacillus horse serum (diaplyte serum) for which we are indebted to Dr. S. R. Douglas of the National Institute for Medical Research, Hampstead, London. The preparations reacted only feebly with anti-BCG rabbit sera. Antigen controls (with normal horse serum, antistreptococcus or antiplague serum) and serum controls were run in all experiments.

The A and S preparations seem to have been comparatively pure from the very beginning, as their serological activity did not increase considerably on prolonged purification. It also should be noted that the S fractions, although containing only half the amount of polysaccharides as the A fractions, were as active immunologically as the latter.

#### *Proteins*

The bacteria (Experiment 1), weighing 60 gm., were first extracted with 0.2 N acetate buffer at pH 4.1, in order to remove the polysaccharides, as described above. The material then was extracted in the cold successively with 500 cc. of 0.2 N acetate buffer at pH 6.2 and with 400 cc. of M/15 phosphate buffer at pH 7.7 and at pH 9.2 respectively. No appreciable amounts of proteins could be precipitated from these solutions by acetic acid or saturation with ammonium sulfate. The residue from the last extraction was digested with 400 cc. of 0.5 per cent aqueous potassium hydroxide



in the cold for 5 days. After centrifugation enough 1 N acetic acid was added to the supernatant liquid to make it acid against litmus. A precipitate formed which was dissolved in 60 cc. of 0.2 N potassium hydroxide solution. The resulting turbid solution was centrifuged at high speed and the protein precipitated with 1 N acetic acid. This precipitation was repeated twice and the protein centrifuged off, washed with acetone, and dried *in vacuo* over phosphorus pentoxide. A light brown powder was obtained which weighed 1.12 gm and gave strong biuret, Millon, and Molisch reactions. This protein contained 1.5 per cent of ash. It had a comparatively weak tuberculin activity.

*Analyses (Calculated on Ash-Free Basis)*—4.994 mg. gave 9.115 mg. of  $\text{CO}_2$ , 3.060 mg. of  $\text{H}_2\text{O}$ ; found, C 49.76, H 6.86

10.131 mg. used 7.77 cc. of 1/70 N HCl; found, N 15.34

9.260 mg. gave 0.800 mg. of  $\text{BaSO}_4$ ; found, S 1.19.

25.170 mg. gave 29.018 mg. of  $(\text{NH}_4)_3\text{PO}_4 \cdot 12\text{MoO}_3$ ; found, P 1.67.

*Optical Rotation*—140.05 mg., dissolved in 10 cc. of 2 N KOH and brought with acetic acid to pH 8.2, had, examined in a 0.5 dm. tube,  $\alpha = -0.35^\circ$ ; found,  $[\alpha]_D^{25} = -50^\circ$ .

Some of the analyses recorded in this paper have been carried out by Dr. A. Elek of the Rockefeller Institute for Medical Research, and by Miss M. Reiner of the Mount Sinai Hospital.

#### SUMMARY

1. Two polysaccharides giving precipitations with anti-BCG horse sera have been prepared from defatted Calmette-Guérin bacilli (BCG).

2. The polysaccharide designated as fraction A is a water-soluble, dextrorotatory, weak acid containing 77.2 per cent of reducing sugars and 2.9 per cent of amino sugars. Its main components are mannose and *d*-arabinose together with a small amount of inositol.

3. The polysaccharide designated as fraction S is insoluble in water and alkali, and soluble in acids. It is a strong adsorption compound between equal parts of a polysaccharide, containing 94 per cent of reducing sugars, and of calcium phosphate.

4. The chemical and immunological properties of a protein obtained from BCG are described.

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## THE AMIDE NITROGEN OF OVALBUMIN

BY AGNES SHORE, HILDEGARDE WILSON, AND  
GEORGE STUECK

*(From the Department of Chemistry, New York University College of  
Medicine, New York)*

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In the hydrolysis of protein by strong acids, the ammonia which is set free constitutes an important fraction of the nitrogen of the protein. Its determination has become part of the routine of protein analysis. From the results of Henderson (1), Gortner and Holm (2), Pittom (3), Van Slyke (4), Vickery (5), and others, however, it has become clear that the amount of ammonia which is obtained depends to some extent upon the conditions of hydrolysis. The curve which relates ammonia production to time is not that of a simple reaction. It appears rather to be the resultant of a rapid process complete in a few hours, and a much slower reaction which continues indefinitely. It is generally assumed that the more rapid reaction consists in the hydrolysis of primary acid amide groups. The secondary evolution of ammonia is usually attributed to such reactions as may be comprehended in the term deamination. If this be the situation, then it follows that precise determination of amide nitrogen can be made only by an analysis of the time course of ammonia production. This was the method adopted by Vickery (5), and is the one which we have used in the present study.

Our observations comprise determinations of the amounts of ammonia formed at intervals during the protracted hydrolysis of ovalbumin in three concentrations of hydrochloric acid (approximately 5, 1, and 0.2 M) and at two temperatures (100° and 85°). The results are summarized in the curves of Figs 1 and 2, wherein ammonia is expressed in equivalents per mole of dry, ash-free ovalbumin. The molecular weight of ovalbumin was assumed to be 34,500.

*Rate of Deamination*—The data included in Fig 1 have been

chosen to illustrate the nature of the deamination process. It will be seen that during the first stages of deamination the rate can be satisfactorily described by a straight line. It appears that the rate of deamination is substantially unaffected by the concentration of acid, and further, that the temperature coefficient is not large. From the slopes of the lines we calculate that the rate at  $100^{\circ}$  is approximately 0.048 equivalent of ammonia per

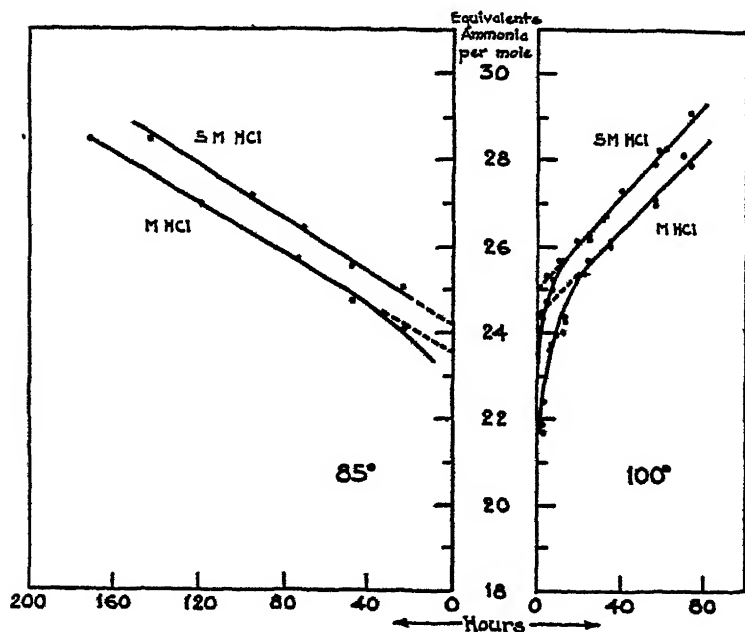


FIG. 1. Deamination at  $100^{\circ}$  and  $85^{\circ}$  in 1 M and 5 M HCl

mole per hour, and at  $85^{\circ}$  is 0.029 equivalent per mole per hour. These correspond respectively to 0.0014 and 0.00085 milli-equivalent per gm. of protein per hour.

*Deamidation*—If both deamination and deamidation are involved in the early part of the process, then extrapolation of the straight line of deamination rate should meet the ordinate at a value corresponding to the equivalents of amide nitrogen in the protein. Actually the extrapolation leads to values which dimin-

ish with the concentration of acid and the temperature. We believe that this is due to the fact that deamination does not become established at its full rate until hydrolysis of the protein is far advanced, and hydrolysis of amides nearly complete. In conformity with this view are the observations that complete solution of the protein in the acid did not occur until the curve approached its straight line phase, and that visible humin formation did not begin until about the same time.

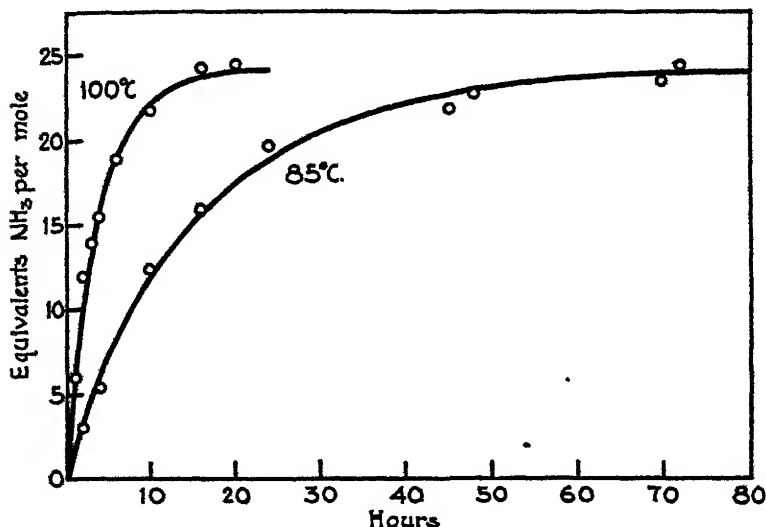


FIG. 2 Velocity of deamidation in 0.2 M HCl at 100° and 85°. The continuous curves are calculated from the mean unimolecular velocity constants, assuming the total amide nitrogen is 24 equivalents per mole. The discrete points are experimental observations

Analysis of Fig. 1 on this basis leads to the conclusion that the probable value for the amide nitrogen lies between 24 and 25 equivalents per mole of protein (0.70 and 0.725 milli-equivalent per gm.). One is tempted to argue that the value of 25 equivalents per mole, which is derived from the curve for 5 M acid at 100°, is the more probable because in this case the extrapolation is so small as to introduce a negligible error. It is doubtful, however, if such precision is justified. Even when due allowance has

been made for deamination, we have still to prove that the value arrived at by extrapolation is a measure of acid amide groups only. This seems beyond definite proof at present. If it could be shown, however, that this phase of the reaction simulated a first order reaction, then there would be presumptive evidence for the conclusion that only one type of group was involved.

The experiments with 1 M and 5 M hydrochloric acid were useless for analysis of the time course of deamidation, because a solid phase was present during the major part of the reaction. It was found that in solutions of hydrochloric acid between 0.1 M and 0.25 M, ovalbumin would remain in solution throughout hydrolysis. Measurements were accordingly made of the rate of the primary phase of ammonia production at 100° and at 85° in 0.2 M hydrochloric acid.

The experimental observations are shown as discrete points in Fig. 2. The continuous lines are unimolecular velocity curves in the calculation of which it was assumed that the total amide nitrogen was 24 equivalents per molecule of protein. The velocity constant used in each case was the mean of the values calculated from the individual observations. We chose 24 rather than 25 equivalents for the amide nitrogen because more consistent constants were calculated from all but the very last few observations at 100° if the lower value was used. In view of the fact that deamination has probably commenced before the completion of deamidation, these later points will be expected to be displaced slightly above the theoretical curve. On the evidence presented one is entitled to conclude that the first stage in the production of ammonia follows a unimolecular curve over more than 90 per cent of its course. The velocity constants found were  $7.15 \times 10^{-5}$  at 100° and  $1.83 \times 10^{-5}$  at 85°, natural logarithms being used and the time being measured in seconds. The value at 85° is subject to some error due to lack of precision in the control of temperature.

The significance of the unimolecular character of this reaction is not clear. We prefer to postpone discussion until the behavior of other proteins has been examined. In the meantime it is legitimate to conclude that all, or almost all, of the ammonia determined by extrapolating the rate of deamination to 0 time originates in one type of group. There can be little doubt that this is the acid amide group. On the basis of all the evidence

we have chosen 24 equivalents per molecule of ovalbumin as the most probable value for the amide groups.

Reviewing the variables of acidity and temperature as they affect the two stages in the production of ammonia, we may conclude that increase in either variable favors deamidation more than deamination. The best conditions for the determination of amide nitrogen would appear to be hydrolysis in a high concentration of acid at a high temperature for a short period. In the case of ovalbumin a substantially correct result is to be expected after 6 to 10 hours at 100° in 20 per cent hydrochloric acid. At the boiling point, the time should be reduced to 3 to 5 hours. In Table I we have assembled from the literature sundry values

TABLE I  
*Various Values for Amide Nitrogen of Ovalbumin*

Author	Conditions	NH <sub>3</sub> per mole <i>equivalents</i>
Osborne and Harris (6)	Boiling 20% HCl, 10 hrs	33 2
" Jones, and Leavenworth (7)	" 20% " 20 "	33 0
Calvery (8)	" 20% " 30 "	34 2
Van Slyke (4)	20% HCl, 100°, 10 hrs	21 3
	20% " 100°, 24 "	23 1
	20% " 100°, 48 "	29 8
Pittom (3)	Boiling strong HCl, see text	24 8
Shore, Wilson, and Stueck	See text	24 0

for the amide nitrogen of ovalbumin. The value attributed to Pittom was calculated by extrapolation from a series of determinations which the author made during a hydrolysis in boiling "strong" hydrochloric acid. The result agrees well with ours. Other values in Table I were made as part of the routine of protein analysis, and are all considerably higher. They emphasize the necessity for control of the error due to deamination. The values attributed to Van Slyke were calculated on the assumption that the protein used by him contained 15.7 per cent nitrogen.

#### EXPERIMENTAL

The experiments reported have been conducted at intervals over a period of 4 years, and four separate preparations of crystalline ovalbumin have been employed. One had been crystallized by



the method of Sørensen (9), the others by a modification of this method in which sodium sulfate replaced ammonium sulfate as the salting-out agent. Three of the preparations were recrystallized three times. One, which had been crystallized once from sodium sulfate, was reprecipitated three times in the amorphous form. The first preparation was coagulated and dried with alcohol and ether; the others were used in the form of dialyzed solutions. In spite of these variations in the method of preparation, all the materials gave the same amide nitrogen within the experimental error of 1 equivalent per molecule. The basis of calculation was the weight of dry, ash-free protein determined experimentally, all measurements of protein solutions being made with a dry pipette calibrated for the weight of protein in the volume of solution which was delivered under standard conditions.

The reaction mixtures, containing about 0.5 gm of protein and the desired concentration of hydrochloric acid, were sealed in glass tubes and placed in the constant temperature bath at 100° or 85°. The mixtures were so prepared that the amount of acid was not less than 10 times the number of equivalents of nitrogen in the protein present. In this way change of pH during the progress of the reaction was controlled. At suitable intervals a tube was removed from the bath, cooled in ice, and its contents transferred to the apparatus in which ammonia was determined.

The alcohol steam distillation method of Foreman (10) was used in the first experiments. We found that this required careful control to prevent slight amide hydrolysis during the distillation, although under proper conditions it gave reliable results. For the majority of the work we abandoned this method for the usual one of distillation *in vacuo*. Instead of using magnesia for the liberation of ammonia from the barely neutralized hydrolysates, we employed a concentrated borate buffer of pH 10. This was done with the object of minimizing alkaline hydrolysis during the distillation.

This work was undertaken at the suggestion of Professor R. K. Cannan, whose interest and advice we wish to acknowledge.

#### SUMMARY

By analysis of the time course of ammonia production during the hydrolysis of crystalline ovalbumin in 5, 1, and 0.2 M hydro-

chloric acid, it is concluded that the probable value for the amide groups is 24 equivalents per mole

The hydrolysis of the amide groups is a first order process. Velocity constants found are  $7.15 \times 10^{-5}$  at  $100^\circ$  and  $1.83 \times 10^{-5}$  at  $85^\circ$ , natural logarithms being used, and with the time measured in seconds.

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## EXPERIMENTS UPON THE EXTRACTION AND STABILITIES OF VITAMIN B ( $B_1$ ) AND OF LACTOFLAVIN

BY BERTHA BISBEY AND H. C. SHERMAN

*(From the Department of Chemistry, Columbia University, New York)*

(Received for publication, September 11, 1935)

The gratifying new knowledge of the chemical natures of vitamin B ( $B_1$ ) and of the lactoflavin factor of vitamin  $B_2$  or G gives added interest to the further questions whether related substances of like nutritional potencies may still be found, and to what extent these vitamins occur free and to what extent in combination with proteins or other substances in plant and animal tissues and secretions. Hence, the experiments upon extractions of natural material with alcohol here recorded, while planned and performed before some of the advances mentioned above had been published, should still be helpful to the fuller working out of the properties of these vitamins or their compounds as they occur in nature, and our results can now be interpreted with more permanent validity than would have been possible before.

The recent work of several investigators has shown that the more heat-stable part of the vitamin B complex can in turn be resolved into two or more factors, one of which is flavin. And the work of Booher, Blodgett, and Page (1) shows that it is the flavin value which is measured by the method of Bourquin and Sherman (2). The work of Sandels on vitamin G (3), in the same laboratory by nearly the same method as that of Bourquin, doubtless dealt with the same factor, and suggested that its stability may be unfavorably influenced by alcohol. The experiments briefly summarized in the present paper deal primarily with the quantitative aspect of extraction by alcohol of the vitamin B ( $B_1$ ) and flavin factors as they exist naturally in milk.

### EXPERIMENTAL

Skim milk powder was the source material here employed. Work published meanwhile indicates that most, but not all, of the

lactoflavin of milk is in a relatively diffusible form and, therefore, presumably either not combined or only loosely combined with the proteins present; that the two now recognized constituents of the vitamin G or  $B_2$  complex are present in milk in essentially the same relative proportions in which they are needed in the nutrition of mammals (4); and that they are not readily separated by ordinary extraction processes, though separable by adsorption (1, 4).

Our starting material had been prepared by the spray-drying of skim milk, in which process the water is evaporated so quickly and at such a low temperature as to make it highly probable that the chemical forms of both vitamins remained essentially as in the fresh milk.

In each of our extraction experiments, 100 gm. of this skim milk powder, after removal of hygroscopic moisture by drying *in vacuo* over sulfuric acid, were mixed with 250 cc. of alcohol (absolute or 80 per cent by weight, and with or without the addition of 1 per cent of glacial acetic acid) and transferred at once to a percolator through which a current of purified nitrogen was flowing. The current of purified nitrogen was continued as a means of constant stirring, as well as of removing any residual air, the percolator being tightly stoppered and sealed with shellac. After 2 hours extraction the stop-cock of the sealed percolator was opened to permit the filtrate (still under purified nitrogen) to pass through paper and cotton on a porous plate, into a distilling flask previously filled with purified nitrogen. The filtration process was so standardized as always to occupy approximately 1 hour, so that the total time of contact of material with solvent should be the same in every experiment. The treatment of the residue with the solvent was then repeated and the second portion of solvent left in contact with the material overnight at room temperature (about 22°), special care being taken that all connections were air-tight. In the morning the extract was filtered off as before and the residue treated twice more with 250 cc. of the solvent. Thus each 100 gm. sample of the dry (nearly fat-free) milk powder was extracted with a total of 1000 cc. of solvent, in four successive operations, during a space of 24 hours, air being carefully excluded by purified nitrogen throughout. (There were also comparative extractions in an atmosphere of oxygen.)

We also performed a series of fractional extraction experiments

in which the treatment as just described was carried through first with absolute alcohol, then followed by the same treatment with 80 per cent alcohol during a second 24 hours.

At the end of each extraction experiment the residue was dried *in vacuo* over sulfuric acid; the extract was concentrated under reduced pressure at a temperature of 21–25°, and then dried upon a regulated amount of corn-starch in a vacuum desiccator, the atmosphere of purified nitrogen being maintained throughout. The corn-starch had previously been dried at 40° in a vacuum oven under nitrogen to free it from occluded air, as recommended by Professor T. C. Taylor whose guidance in the handling of the starch is gratefully acknowledged. In view of the work done meanwhile in other laboratories upon lactoflavin, it should be explained that the extraction experiments were carried out in ordinary or Pyrex glass containers in a room which was lighted through thick glass windows in the daytime and was entirely dark at night, and the preparations were stored in a dark refrigerator during the subsequent feeding experiments. Also, heavy denim covers were placed over desiccators containing material which was being dried or stored *in vacuo* or under diminished pressure. Thus the possible influence of visible light upon the lactoflavin was not entirely precluded but was very considerably minimized.

In all cases identical preparations were fed for vitamin B ( $B_1$ ) and for flavin values, the feeding experiments following immediately upon the extraction experiments, the latter being repeated weekly so that the preparations should be only from 1 day to 1 week old as fed (the preparations were stored during the week of feeding in a dark refrigerator at about 10°).

Each measurement of a vitamin value involved the feeding of from four to ten experimental animals at the level of intake which was found suitable for the making of quantitative comparisons as in the experiments of Chase and Sherman (5) for the determination of vitamin B ( $B_1$ ); and of Bourquin and Sherman (2) for "vitamin G," *i.e.* for flavin values. Our experience in the determination of vitamin B has confirmed both that of Chase and Sherman (5) and that of Williams, Waterman, and Gurin (6), showing that the two sets of findings are not in conflict, but rather are supplementary to each other. The method of autoclaving used by Chase and Sherman is effective in destroying the vitamin B ( $B_1$ )

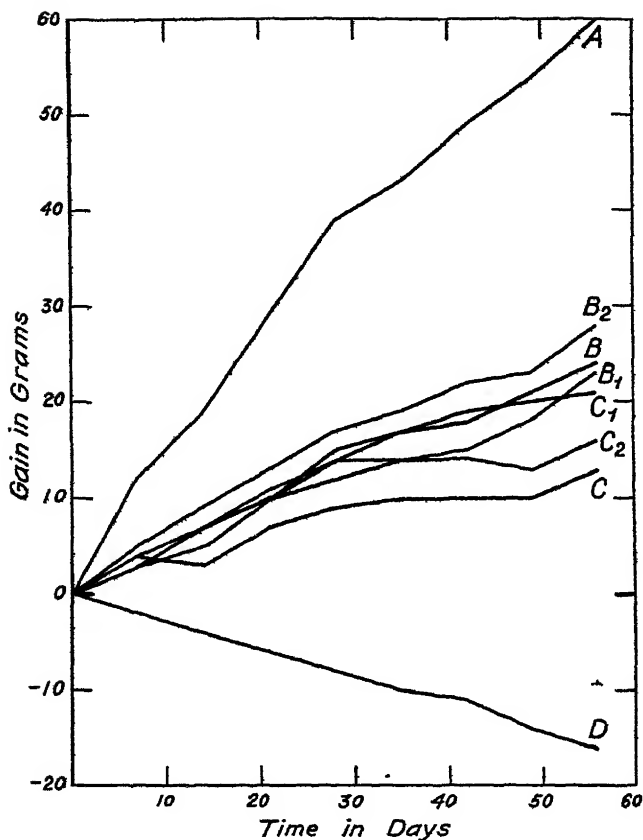


FIG 1 Average weight curves of test animals comparing flavin values of 80 per cent alcohol extracts and residues of skim milk powder. Extractions made (1) in an atmosphere of purified nitrogen (Curves B,  $B_1$ ,  $B_2$ ) and (2) in an atmosphere of oxygen (Curves C,  $C_1$ ,  $C_2$ ). The weekly allowance was 3.6 gm. of skim milk powder, for the positive controls; or for each test animal a corresponding amount of an extract or a residue; or a mixture of extract and residue, one-half the amount of each. The individual curves represent the results of feeding: Curve A, skim milk powder (positive controls); Curve B, extract made under nitrogen; Curve  $B_1$ , residue from extract made under nitrogen; Curve  $B_2$ , mixture of extract (B) and residue ( $B_1$ ); Curve C, extract made under oxygen; Curve  $C_1$ , residue from extract made under oxygen; Curve  $C_2$ , mixture of extract (C) and residue ( $C_1$ ); Curve D, basal diet only (negative controls).

of yeast of the type which they employed; while the different type of yeast encountered by Williams, Waterman, and Gurin requires a more drastic treatment because of its acidity and its higher initial content of the antineuritic vitamin. Our measurements of vitamin values were guided by this experience and in other details by the experience of other colleagues; but our feeding methods were essentially as described in other papers from this laboratory. The accounts and detailed data of the assays of the extracts and residues may, therefore, be omitted for the sake of brevity and the findings may be summarized with the aid of a single chart (Fig. 1).

#### SUMMARY

The vitamin B ( $B_1$ ) value was found to be well conserved throughout the extractions and drying of extracts made as described, whether the solvent were neutral or acidulated alcohol, absolute or of 80 per cent by weight. Practically all of this vitamin (in the form found in milk) was extracted by 80 per cent alcohol, and practically none of it by absolute alcohol, under the conditions described. Acidulation of the alcohol with 1 per cent of glacial acetic acid had no measurable effect upon its solvent effect in this case. Additive results were obtained with mixtures of extract and residue, indicating no vitiation of the feeding method by lack of any "new" or unknown factor.

Notwithstanding its well known greater stability to heating, the vitamin G or flavin value was apparently somewhat less completely conserved than that of vitamin B. That oxidation by residual or occluded air is not a very destructive influence in extractions of this sort may be seen from Fig. 1. In view of this fact and the recent findings of Supplee, Ansbacher, and Bender (7) the diminution of flavin value was most probably due to the combined effects of the visible light which penetrated the glass containers in which the extractions were performed, the small amount of residual oxygen, and possibly a slight influence of the alcohol present.

Under the conditions described, the flavin value of milk powder was not measurably extracted by absolute alcohol at room temperature; by 80 per cent alcohol at room temperature it was less completely extracted than was the vitamin B ( $B_1$ ) value, about



equal amounts of the flavin appearing in the extract and in the residue as seen in Fig. 1. When these results are compared with those of other workers, it would appear that the solubility of lactoflavin in alcohol increases rather rapidly with temperature. The extraction of flavin from milk powder was not measurably influenced by acidulating the alcohol with 1 per cent of glacial acetic acid. Quantitative feeding experiments with mixtures of extract and residue gave no indication of any measurable separation of essential factors; but rather confirmed the evidence of other experiments that measurements of vitamin G values as hitherto made by the Bourquin method are essentially measures of flavin values—whether of the natural food or of such extracts and residues as are here described

While all estimates of relative amounts and partial losses of the vitamins here discussed are based upon the quantitative data of the weight curves, the identities of the factors dealt with were sufficiently verified by frequent observations upon the symptoms of nutritional deficiency in the experimental animals employed.

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## ON THE PLASTID PIGMENTS OF MARSH DODDER

By G. MACKINNEY\*

(From the Carnegie Institution of Washington, Division of Plant Biology,  
Stanford University, California)

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The dodders, comprising the genus *Cuscuta* in the family Convolvulaceæ, are described by Jepson (1) as, "Annual leafless parasites, destitute of green color, with twining filiform stems. . . . The germinating seed produces a twining stem; this becomes parasitic by means of suckers which penetrate the bark of the host." Two species have been examined, *Cuscuta subinclusa* (which parasitizes chaparral and poison oak) and *Cuscuta salina*, the marsh dodder (found on saline herbs). The color of the former is a pale straw with a faint greenish tint.<sup>1</sup> That of the latter is golden, changing to a fiery red as the season advances. A restricted region at the base of the minute white flower is green. By sectioning the flowers, one may observe that the green region is definitely localized—in the bud, around the vascular tissue about the receptacle; in the mature flower, to the ovules and maturing fruit. The under surface of a mass of marsh dodder may also appear greenish.

The lack of pigmentation and sparse distribution in this locality of the former species made detailed study impossible, though there is apparently no major difference in the pigment complex of the two species.

*Detection of Chlorophyll*—When 5 gm. of the flowering portions were macerated with 95 per cent alcohol, a filtered extract gave sharply defined absorption bands with maxima at 666  $m\mu$  and 620  $m\mu$ , with the end absorption commencing at about 520  $m\mu$ . In an equivalent extract from the stems, the prominent red absorption band of chlorophyll was barely discernible; chlorophyll was similarly detected in *Cuscuta subinclusa*.

\* National Research Council Fellow.

<sup>1</sup> The writer is informed it may appear definitely green when growing in the shade.

*Carotenoid Pigments*—Examination of crushed sections of the stems under the microscope shows the reddish coloring matter to be segregated in small bodies, plastid-like in appearance.

During the isolation of  $\alpha$ - and  $\beta$ -carotenes from dodder, from a petroleum ether crude extract (2), it was noticed that the bulk of the color was due to pigments more strongly adsorbed than either of these components. In this experiment, a total fresh weight of 16.0 kilos of dodder was picked, and one-half of the  $\alpha$ - and  $\beta$ -carotene was worked up, to yield 99.0 mg. of recrystallized carotene, or 12.5 mg. per kilo (2). By washing the column of adsorbent (35 cm.  $\times$  6.5 cm. in diameter) with petroleum ether, by the method of Strain (2, 3), there were left on the column, after elution of the  $\alpha$ - and  $\beta$ -carotenes, three wide reddish colored zones, surmounted by a greenish zone of chlorophyll or its derivatives. These reddish zones were partially separated by successive washings with benzene (2 liters) and dichloroethane (3 liters).

From the band immediately succeeding the  $\beta$ -carotene, 20 mg. of a pigment identified as  $\gamma$ -carotene were obtained (m.p. 164–165° corrected; absorption spectra maxima, in ethanol at 489 and 460  $m\mu$ , in carbon disulfide at 530, 496, and 471  $m\mu$ ). From the next band approximately 2 mg. of lycopene were isolated (absorption maxima, in carbon disulfide at 544, 507, and 472  $m\mu$ ). From the most strongly adsorbed band, 8 mg. of rubixanthin were obtained (m. p. 168–169° corrected; absorption maxima in carbon disulfide at 529, 495, and 470  $m\mu$ , indistinguishable from  $\gamma$ -carotene). Values for  $\epsilon$ , the molar absorption coefficient, were obtained for samples of  $\gamma$ -carotene and rubixanthin (Table I) in carbon disulfide.

Identification of these pigments has been based upon their two most characteristic properties, behavior on an adsorbent, and absorption spectra. It may be doubted whether, in view of their low melting points, a purity greater than 90 to 95 per cent has been attained. From the work of Hausser (4), it may be anticipated that the absorption coefficients of  $\gamma$ -carotene and rubixanthin should be intermediate between those of  $\beta$ -carotene and lycopene, as they contain one more conjugated double bond than the former, and one less than the latter. The absorption coefficients are shown to be in agreement with this intermediate position.

The difficulties of purification are due to apparently abnormal

concentrations of waxes, presumably related to conservation of water in a halophytic environment. Strain (5) has shown that a wax-like hydrocarbon may precede the  $\alpha$ -carotene with little or no adsorption. A similar substance is present in dodder, in such large amounts (18 gm. were isolated in crystalline form) that it was impossible to separate it effectively from the 99 mg. of  $\alpha$ - and  $\beta$ -carotenes solely by adsorption technique. The lycopene band was likewise exceptionally high in colorless impurities.

It was recognized that the low yields of the more strongly adsorbed pigments were in part due to impurities, but more especially to the length of the time they were adsorbed on the column when petroleum ether was used. An effective separation into four main

TABLE I  
*Molar Absorption Coefficients in Liters per Mole Cm.*

Pigment	$\epsilon \times 10^{-4}$		No. of conjugated double bonds
$\beta$ -Carotene*	10 42 at 483 $m\mu$	9 24 at 512 $m\mu$	11
$\gamma$ -Carotene	10 78 " 497 "	9 71 " 529 "	12
Rubixanthin	11 40 " 495 "	10 24 " 529 "	12
Lycopene*	13 6 " 508 "	12 6 " 544 "	13

\* Included for comparative purposes, from observations made at that time. For absolute values, and discussion, see the paper by Smith (Smith, J. H. C., presented before the Organic Division at the meeting of the American Chemical Society at San Francisco in 1935).

bands has been attained with petroleum ether containing 15 to 20 per cent dichloroethane. Under such conditions, neither the lycopene nor  $\gamma$ -carotene bands appear homogeneous, but it has not been possible to obtain eluted solutions in which the lycopene and  $\gamma$ -carotene spectra do not mask additional components. Inasmuch as some chlorophyll is found in dodder, one may surmise that traces of the more commonly occurring leaf xanthophylls are also present. As an instance of the improvement in yields, by reduction of the time factor, the following experiment may be cited. A 75 cc petroleum ether-concentrated extract (from 0.24 kilo of meal, 1 65 kilos fresh weight) was passed over a column of magnesia (37 cm  $\times$  6 5 cm in diameter) and washed with approxi-

mately 2 liters of petroleum ether containing 15 per cent dichloroethane. Four main bands were obtained. Owing to the presence of the dichloroethane, there was virtually no separation of  $\alpha$ - and  $\beta$ -carotene from the wax-like substances, and this fraction was washed through. The column was then mechanically divided, and each colored zone was eluted with pyridine (which was found to be even more satisfactory than alcohol for removing strongly adsorbed pigments). The solutions were rapidly concentrated to 3 to 5 cc., to which double the quantity of absolute ethanol was added, and the pigments were allowed to crystallize.

Yields of 4 to 5 mg. of crude rubixanthin, 8 to 9 mg. of lycopene, and 36 mg. of  $\gamma$ -carotene were obtained. These represent, per kilo of fresh weight, 2 to 3 mg. of rubixanthin, 4 to 6 mg. of lycopene, and 20 to 25 mg. of  $\gamma$ -carotene. The  $\alpha$ ,  $\beta$ -carotene content has already been reported, 12.5 mg. per kilo (2)

#### SUMMARY

In an investigation of the plastid pigments of marsh dodder, it is shown that chlorophyll is present, though in low concentration, except possibly in sharply localized regions.  $\alpha$ - and  $\beta$ -carotenes,  $\gamma$ -carotene, lycopene, and rubixanthin have been isolated. An unusually high concentration of unsaponifiable colorless material is encountered, which hinders a rigorous purification of the various components. Identification of the  $\gamma$ -carotene and rubixanthin is based on their two most characteristic properties, behavior on an adsorbent and absorption spectra.

The dodder represents the richest source of  $\gamma$ -carotene known to the writer, readily available in California.

Acknowledgment is made to Dr. H. A. Spoehr and all members of the Division for advice and suggestions

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## A CRYSTALLINE BY-PRODUCT OBTAINED IN THE LARGE SCALE EXTRACTION OF THEELIN AND THEEOL

By A. W. DOX, W. G. BYWATER, AND F. H. TENDICK

(From the Research Laboratories of Parke, Davis and Company, Detroit)

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In the commercial extraction of theelin and theelol from human pregnancy urine, an acidic, nitrogenous, crystalline substance may be separated from the crude extract. Preliminary Allen-Doisy tests showed this compound to be estrogenically inert. The constant occurrence of this substance (about 4 mg. per gallon of urine) suggested that a precursor of theelin or theelol had been carried through the process. If such were the case, a simple acid or alkaline hydrolysis to theelin or theelol should then be possible. However, the by-product was resistant to hydrolysis. The high decomposition temperature, extreme insolubility in ordinary organic solvents, and the relative stability of the substance indicate a hitherto unknown constituent of pregnancy urine.

The crystalline by-product is practically insoluble in neutral organic solvents. It is characterized by the formation of an intense blood-red color when dissolved in cold concentrated sulfuric acid and by a green fluorescence when dissolved in alkali. Carbon dioxide reprecipitates the substance from its alkaline solution in characteristic diamond-shaped crystals (Fig. 1). Fig. 2 shows the typical crystals obtained by dilution of the pyridine solution with ether. Qualitative diazo, nitroprusside, murexide, biuret, and ninhydrin tests are negative. Color reactions for tryptophane, mono- and disubstituted guanidines (1), histidine, xanthine, and hypoxanthine are also negative. The  $\beta$ -naphthol color test after diazotization treatment with hydrochloric acid and sodium nitrite is likewise negative. A 7.5 per cent solution in pyridine shows no optical rotation. Combustion and micro-Kjeldahl analyses give as the most probable formula  $C_{21}H_{23}O_3N_4$ . This formula is in

agreement with the neutralization equivalent calculated from the sodium content of the monosodium salt, and with the analysis of



FIG. 1 Theelin by-product ( $C_{21}H_{22}O_5N_4$ ) precipitated from an alkaline solution with carbon dioxide



FIG. 2 Theelin by-product ( $C_{21}H_{22}O_5N_4$ ) crystallized from pyridine-ether

the alkali-insoluble monoethyl derivative Zerewitnoff analysis shows 3 active hydrogen atoms on the basis of molecular weight 378.

These results and a positive pyrrole pine-splinter reaction (on heating the solid) suggest a compact nitrogen heterocyclic ring system. Chromic acid oxidation gives a crystalline acid of the most probable formula  $C_{18}H_{18}O_7N_2$ . Esterification of this acid with diazomethane in methyl alcohol yields an alkali-insoluble product containing, however, a greater percentage of nitrogen than the acid itself.

Injected intraperitoneally in white mice the substance is relatively non-toxic. No gonadotropic, thyrotropic, nor lactogenic activity could be demonstrated. The estrogenic activity is less than 5 rat units per mg. hypodermically and less than 1 oral unit per mg. It is possible that this constituent of gravid urine is a metabolic product of the blood or bile pigments containing four pyrrole nuclei and may be a constituent of normal urine which has hitherto escaped observation. The possibility that the substance is an excretory product of some commonly used drug is still more remote

#### EXPERIMENTAL

*Isolation and Purification of By-Product*—The crude extract obtained from the large scale extraction of pregnancy urine by the Doisy method was dissolved in hot absolute alcohol. This solution when cooled yielded the nitrogenous compound, leaving the theelin and theelol. Purification was effected by dissolving 25 gm. of the original precipitate in 450 cc. of hot alcohol and 25 cc. of 25 per cent sodium hydroxide solution, filtering, then precipitating by adding a solution of 12 cc. of glacial acetic acid in 300 cc. of alcohol and 200 cc. of water. A similar procedure was used for recrystallization. The substance decomposes when heated in a capillary tube to  $360^\circ$ .

<i>Analysis</i> — $C_{21}H_{22}O_8N_4(378)$ .	Calculated.	C 66.66, H 5.81, N 14.82
	Found.	" 66.73, " 5.61, " 15.20
		" 66.84, " 5.59, " 15.26

Qualitative tests for the halogens, arsenic, sulfur, and phosphorus were negative.

*Molecular Weight Determination*—The compound was converted to the sodium salt by dissolving 1.5 gm. in slightly less than the calculated quantity of N sodium hydroxide, filtering the undis-



solved material, and evaporating the filtrate to dryness. The sodium was weighed as sodium sulfate after ignition. The neutralization equivalent by this method was 362. The sodium content was 5.96 per cent, while the theory for  $C_{21}H_{21}O_3N_4Na$  is 5.75 per cent. By titrating the excess standard alkali used to dissolve the substance in alcohol, the neutralization equivalent was found to be 357 (precipitation) and 364 (methyl red). These results, as does the formation of a monoalkyl derivative, indicate a mono-acidic compound. With 378 as the molecular weight, Zerewitinoff analyses gave 2.88 and 2.85 active hydrogen atoms, only one of which was acidic enough to react with sodium hydroxide. The Rast micro- or semimicromolecular weight determination was not feasible, since the compound is insoluble in molten phenol, camphor, urethane, and naphthalene.

*Characterization. Color Reaction with Sulfuric Acid*—1 mg. of the by-product produced a blood-red color in 1 cc. of cold concentrated sulfuric acid. Warming the solution deepened the color and caused the appearance of a green fluorescence; when diluted, the solution turned brown and a precipitate separated. Neither theelin nor theelol gave the color with cold sulfuric acid. The Zimmermann reagent (*m*-dinitrobenzene and alkali) for the  $CH_2CO$  grouping gave only a brown color on prolonged standing, whereas a control test with theelin gave an immediate violet color (2).

*Preparation of Monoethyl Derivative*—Ethylation was effected by dissolving 1.5 gm. of the substance in 25 cc. of alcohol with an excess of 50 per cent sodium hydroxide, then adding 3 cc. of ethyl sulfate. During the following half hour of mechanical shaking some solid separated and the solution became acidic. An excess of strong base was again added with 2 cc. of ethyl sulfate and the shaking resumed. The treatment was repeated a third time, then the suspension was diluted with 25 cc. of water, made strongly alkaline with 4 per cent sodium hydroxide solution, and filtered. The yield was nearly quantitative. The ethyl derivative was recrystallized from dilute alcohol in pale yellow crystals melting at 277–278° (darkening at 275°).

<i>Analysis</i> — $C_{21}H_{21}O_3N_4(C_2H_5)$	Calculated.	C 67.99, H 6.41, N 13.79
	Found	" 68.31, " 6.38, " 13.66
		" 68.52, " 6.46, " 13.66

The acetyl number is 0. This result may be due to the extremely low solubility of the substance in acetic acid-acetic anhydride. Furthermore, the failure to react because of insolubility may account for the quantitative recovery of unchanged material after treating an ether suspension with diazomethane.

*Action of Hydrolytic Agents*—Dilute and concentrated hydrochloric acid and concentrated hydrobromic acid did not hydrolyze the substance. During the prolonged refluxing to which the suspensions were subjected, a green fluorescence developed in the solutions; otherwise no reaction was detected. When 500 mg. in 25 cc. of 25 per cent alcoholic potassium hydroxide were warmed, the characteristic green fluorescence appeared. The top of the reflux condenser was connected with a flask containing standard acid. During 7 hours refluxing 2.9 mg. or 0.5 per cent of ammonia or volatile amine was collected. The recovered product (80 per cent) possessed the characteristics of the original material.

*Chromic Acid Oxidation*—During the oxidation of 5 gm. with an excess of chromic acid in acetic acid a crystalline solid separated, which was filtered out. The filtrate was made alkaline and distilled. The volatile base was identified by analysis as ammonia.

The yield of solid acid was 1.2 gm. It possessed no definite melting point but turns a bright scarlet at about 253°, which deepens until the sample is black at 269°. Analysis of the sample gives for the most probable formula  $C_{18}H_{18}O_7N_2$  (C 57.75, H 4.81, N 7.49; found, C 58.02, 57.70, H 5.05, 5.18, N 7.74, 8.00). The observed neutralization equivalent was 258 (in alcohol). Too much reliance cannot be placed in this value, since it is known that nitrogen-containing acids give abnormally high results. Esterification was consequently attempted to determine the number of carboxyl groups present. With an excess of diazomethane an alkali-insoluble product was isolated whose nitrogen content was greater than that of the starting material (found, N 8.68 and 8.68).

*Physiological Action*—The theelin by-product was first examined for estrogenic activity by the Allen-Doisy test and was found to have less than 5 rat units per mg. by hypodermic injection and less than 1 unit orally.

Toxicity tests were made with white mice. A 2.5 per cent stock solution of the substance in 2 equivalents of alkali was injected intraperitoneally. A dosage of 0.0003 gm. per gm. of body weight

caused no visible effect, while 0.00055 gm. per gm. was lethal. In anesthetized dogs 1.0 cc. and 2.0 cc. of the stock solution produced slight transient depressor action with no indication of a definite effect on the heart or peripheral blood vessels.

Although little is known of the actual structure of the by-product, its presence in pregnancy urine and its high nitrogen content raised the question of the relationship of this unknown substance to the gonad-stimulating factor present in such urine. Consequently the gonadotropic activity was investigated. Even at a very high dose of 6 mg. no evidence of ovarian stimulation was noted.

During routine assay of extracts of the anterior pituitary, the theelin by-product was examined for lactogenic and thyrotropic activity. The results were entirely negative.

#### SUMMARY

1. An alkali-soluble crystalline by-product has been obtained in large scale extraction of human pregnancy urine. The probable formula for this substance is  $C_{21}H_{22}O_3N_4$ . The compound is resistant to hydrolysis, and gives no reaction for proteins, amino acids, or purines. Zerewitinoff determinations show the presence of 3 active hydrogen atoms, 1 of which is easily ethylated to give a monoethyl, alkali-insoluble derivative.

2. Chromic acid oxidation yields an acid,  $C_{18}H_{18}O_7N_2$ .

3. Preliminary biological assay of the substance shows no hormone activity although a toxicity to mice in doses of 0.00055 gm. per gm.

The authors are grateful to Dr. D. A. McGinty and to Mr. L. W. Rowe for the biological assays.

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# THE EFFECT OF HYDRAZINE ON THE PRODUCTION OF ACETONE BODIES IN THE PHLORHIZIN-INTOXICATED ANIMAL

By MAX M. GREENBERG

(From the Laboratory of Biological Chemistry, School of Medicine, University of Texas, Galveston)

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Whereas hydrazine has a tendency to deplete an organism of its carbohydrate reserves (1, 2) and produce hypoglycemia (1, 3, 4), investigators have often been surprised at the lack of recognizable amounts of ketone bodies in the urine of hydrazine-intoxicated animals (5, 6). Wells (7) has shown that hydrazine attacks the parenchymatous cells of the liver exclusively,<sup>1</sup> so, in view of the increasing evidence that the ketone bodies are formed chiefly in the liver (8, 9), it appeared worth while to investigate more closely the production of acetone bodies in the hydrazine-intoxicated animal. This paper is an account of such an investigation.

## EXPERIMENTAL

Because of the well known resistance of the lower animals to fasting ketosis ((10) p. 667) (11), and in order to obtain information on the utilization of glucose, it was thought better to study the formation of ketone bodies in the fasting phlorhizinized animal before and after hydrazine administration than to study it in the normal fasting animal before and after hydrazine. In order to eliminate any effect on ketone body production, which might arise from the vomiting which hydrazine is known to produce in most mammals, it was decided to use rats as the experimental animals.<sup>1</sup>

In every case male rats of about 350 to 400 gm. were used. Each animal was phlorhizinized for a period of 3 days and the output in the urine of acetone bodies, total nitrogen, amino acid

<sup>1</sup> Lewis and Izume (2) found that, in the rabbit, large amounts of hydrazine may also produce slight injury to the kidneys

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nitrogen, and sugar determined. After a lapse of time, sufficient to insure recovery (generally 2 weeks), the same animals were phlorhizinized and hydrazinized for another period of 3 days and the urine analyses repeated. To control the series more accurately the order of the periods of hydrazine and phlorhizin intoxication and of phlorhizin intoxication only were reversed in a few cases.

Each period started at 9 a.m. All food was removed from the cages at 5 p.m. of the previous day, but water was permitted *ad libitum*. Phlorhizin intoxication was produced by daily subcutaneous injections of 25 mg. of phlorhizin in olive oil. To produce mild hydrazine intoxication the animals were injected subcutaneously with 40 mg. of hydrazine sulfate at 9 a.m. of the 1st day and 25 mg. of the drug at 4 p.m. of the 2nd day of the period. The hydrazine salt was administered in aqueous solution of 2.5 per cent concentration.

Urines were collected in the manner suggested by Levine and Smith (11), under paraffin oil, and preserved with sodium fluoride. The funnels were washed daily with 1 per cent sodium fluoride solution, and the washings added to the urines.

Total acetone body determinations were made by the gravimetric method of Van Slyke and Fitz (12). Determinations of sugar were made by the fermentation method of Somogyi (13), with the Shaffer-Hartmann (14) microcarbonate reagent. Total nitrogen was estimated by the regular Kjeldahl method and amino acid nitrogen by Folin's (15) method.

In a few cases total blood acetone bodies, non-protein nitrogen, and whole blood carbon dioxide capacities were determined for the last day of each period. Behre and Benedict's (16) method was used for the determinations of acetone bodies in the blood. Non-protein nitrogen was estimated by the micro-Kjeldahl method of Folin and Wu (17), and carbon dioxide capacity was determined with the Van Slyke (18) direct volume apparatus. The blood was drawn directly from the heart under nembutal anesthesia, 4 mg. of the drug being used per cg. of body weight.

### DISCUSSION

In the ensuing discussion, for the sake of convenience, the period of combined fasting, phlorhizinization, and hydrazine

intoxication will be called the experimental period, and the period of fasting and phlorhizin intoxication, only, will be called the control period.

Experiments were done on seven animals. In all of them the excretion of acetone bodies was markedly greater during the control period than during the corresponding experimental period, the output of ketone bodies during the control period being 2 to 19 times that of the corresponding experimental period (Table I, Column 2). Moreover, despite a marked variation in the excretion of acetone bodies among the animals, in no case did the maximal excretion in the experimental periods exceed the minimal amount excreted by any animal in its control period. Although there is a probability that the animals were not always completely phlorhizinized, yet these results are sufficiently conclusive to indicate that the output of acetone bodies is markedly lessened by hydrazine liver injury. The observations are in agreement with the experiments of Fischler and Kossov (19), in which a decreased excretion of ketone bodies was observed in the phlorhizinized dog after an Eck fistula was made, and the findings of Himwich, Goldfarb, and Weller (8), that the liver is the chief site of production of acetone bodies.

Analyses, made on the last day of each period in a few cases, show that blood acetone bodies were about twice as high during the control periods as during the experimental periods (Table II, Column 5), carbon dioxide capacities half to two-thirds as great in the control as in the experimental periods (Table II, Column 7), and non-protein nitrogen, although high, essentially the same in both periods (Table II, Column 6). These blood ketone body determinations, together with the determinations of alkali reserve, refute, as do the non-protein nitrogen determinations, any assumption that the decreased excretion of acetone bodies during hydrazine intoxication is due to retention of acetone bodies by the kidneys.

The amounts of urinary amino acid nitrogen indicate that the decreased excretion of acetone bodies is not attributable to decreased deamination of ketogenic amino acids. In three cases, Rats R-109, R-113, and R-114, the output of amino acid nitrogen was essentially the same during the control and experimental periods, in one case, Rat R-116, it was definitely higher during the

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TABLE I  
*Urinary Constituents before and after Hydrazine*

Rat No	Period	Average weight	Urinary acetone bodies as acetone	Urinary N	Glucose possible from protein*	Urinary glucose	Glucose oxidized†	Amino acid N
		(1)	(2)	(3)	(4)	(5)	(6)	(7)
		gm	mg	gm	gm	gm	gm	mg
R-108	Control	285	355	0.783	2.84	1.65	1.19	
	Experimental	285	61	0.504	1.83	1.60	0.23	
R-109	Control	320	302	0.563	2.04	1.62	0.42	22
	Experimental	380	46	0.577	2.09	1.75	0.34	21
R-112	Control	450	434	0.790	2.87	1.80	1.07	
	Experimental	436	102	0.777	2.82	1.76	1.06	
R-113	Control	350	488	0.570	2.07	2.63	-0.56	34
	Experimental	380	178	0.660	2.39	1.85	0.54	24
R-114	Control	400	180	0.725	2.63	1.34	1.29	29
	Experimental	430	86	0.615	2.23	2.08	0.15	37
R-115	Control	383	972	0.874	3.17	2.53	0.64	106
	Experimental	425	155	1.047	3.79	0.53		31
R-116	Control	370	380	0.584	2.12	1.82	0.30	17
	Experimental	425	20	0.643	2.33	1.20	1.13	48

\* Calculated from the urinary nitrogen (Column 3) by multiplying by 0.25 and 0.58 according to the method of Lusk (10).

† Calculated by subtracting the values of Column 5 from the values of Column 4.

TABLE II  
*Blood Acetone Bodies, Non-Protein Nitrogen, and Alkali Reserve in Phlorhizinized Rat before and after Hydrazine*

Rat No	Period	Average weight	Urinary acetone bodies	Blood ketones as acetone	Blood non-protein N	Whole blood CO <sub>2</sub> capacity
(1)	(2)	(3)	(4)	(5)	(6)	(7)
		gm	mg	mg per cent	mg per cent	vol per cent
R-109	Control	320	302	65	173	20
	Experimental	380	46	35	144	44
R-115	Control	383	972	82	60	32
	Experimental	425	155			45
R-116	Control	370	384	34	46	30
	Experimental	425	20	17	64	45

experimental period, and in one case, Rat R-115, it was definitely higher in the control period.

Since the levels of nitrogen and glucose excretion did not change in the control and experimental periods in the two animals, Rats R-109 and R-112 (Table I), it appears that the decreased excretion of acetone bodies during hydrazine intoxication is independent of any increase in protein or glucose metabolism in these animals. The variation in the excretion of nitrogen and glucose in the control and experimental periods of the other animals prevents any cursory conclusions concerning the degree of glucose and protein metabolism in the two periods of these animals. However, since the glycogen content of rats fasted 16 hours is small (20) and practically a constant percentage of the body weight (21), the difference between the amounts of glucose available from the protein metabolized and the amounts of glucose excreted constitutes a relative measure of the quantities of ketolytic materials oxidized in the two periods in these animals. Such calculations, of course, are based on the assumption that the ability of the phlorhizinized-hydrazinized rat to store glycogen is not much different from that of the phlorhizinized rat. This assumption is probably true in general, because both phlorhizinized animals ((10) p. 322) and hydrazinized animals (6) store very little glycogen. Such calculations (Table I) indicate that the amounts of ketolytic materials oxidized were greater in the control than in the experimental periods in Rats R-108 and R-114, and less in the control than in the experimental periods in Rats R-113 and R-116. Judging from the glucose excreted during the experimental period, Rat R-115 was only slightly phlorhizinized at the time, and, hence, the result is disregarded.

Although Underhill and Murlin (22) have reported that a slight increase in the respiratory quotient of fasting dogs occurs after the administration of hydrazine, Izume and Lewis (6) believe that hydrazine does not increase the rate of glucose metabolism. The present investigation apparently supports the contention of Izume and Lewis. At the same time it brings out the fact that hydrazine causes a decrease in the production of acetone bodies, which is independent of any decrease in deamination of (ketogenic) amino acids or any increase in glucose or protein metabolism.



## 436 Acetone Body Production after Hydrazine

### SUMMARY

1. It was observed that phlorhizinized, fasting rats excrete less acetone bodies after hydrazine intoxication than before.

2. The decrease in the excretion of acetone bodies in these animals was found to be independent of any decrease in deamination of (ketogenic) amino acids or any increase in the metabolism of glucose or protein.

The author is indebted to Dr. B. M. Hendrix for helpful suggestions.

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## BIXIN SOLUTIONS AS COLORIMETRIC STANDARDS FOR THE DETERMINATION OF CAROTENE

BY HARRY N. HOLMES AND WERNER H. BROMUND

(From the Severance Chemical Laboratory, Oberlin College, Oberlin)

(Received for publication, September 16, 1935)

The Willstatter-Stoll method of quantitative estimation of carotene in petroleum ether solutions is advocated by Palmer (1) who objects to standards made from carotene in organic solvents because of their rapid deterioration.

*Determination of Carotene in Petroleum Ether*—For dilute solutions of carotene in petroleum ether,  $K_2Cr_2O_7$  dissolved in water to make a 0.2 per cent solution was found by Willstatter and Stoll to give an excellent color match. The following formula, as worked out from data given by Palmer, is convenient in calculating the per cent concentration of carotene in an unknown petroleum ether solution.

$$\text{Per cent carotene} = 0.00268\% \times \frac{\text{depth of carotene solution equivalent to standard}}{\text{depth of unknown solution}}$$

In our laboratory, with a Leitz dipping colorimeter, it seemed best to dilute the standard and unknown solutions so that the color or intensity matches could be restricted to a depth of from 15 to 25 mm. Color matching was found to be most accurate within this range.

Carotene may be obtained from the S. M. A. Corporation, Cleveland, or prepared in convenient quantities by the method of Holmes and Leicester (2).

*Determination of Carotene in Benzene*—It was observed in our laboratory that the 0.2 per cent  $K_2Cr_2O_7$  water solution standard used for the determination of carotene in petroleum ether was not at all suitable as a standard for the determination of concentration

of carotene in other solvents, such as benzene and chloroform. Because of the high refractive indices of these solvents, the color of the solutions was shifted toward the red end of the spectrum, and a suitable color match with dichromate solution was found to be impossible. It was discovered, however, that bixin in benzene, diluted to the proper concentration, gave a color which matched very well that given by carotene in benzene. Since bixin in ben-

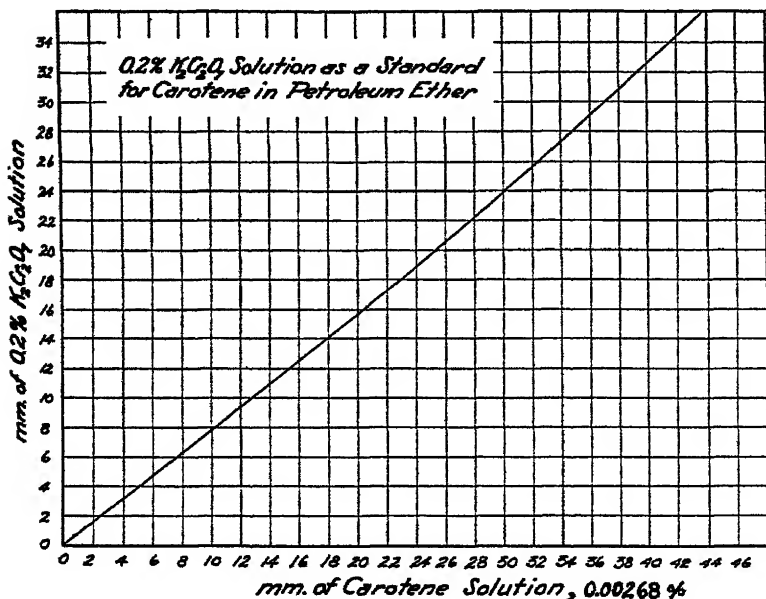


FIG. 1 Colorimetric comparison of a 0.00268 per cent solution of carotene in petroleum ether with a standard 0.2 per cent  $K_2Cr_2O_7$  aqueous solution

zene is comparatively stable and since crystalline bixin can readily be prepared by the method of Holmes and Bromund (3) such a standard is of very practical use

If carotene of 100 per cent purity is not available, a standard solution of carotene in benzene may be prepared by the more indirect method of making a solution of nearly pure carotene in petroleum ether, determining its concentration by the dichromate standard with reference to Fig. 1, removing the petroleum ether by a quick distillation under reduced pressure to prevent decom-

position, and then dissolving the residue in a known volume of pure benzene. The benzene solution thus obtained is of a known concentration and can be compared colorimetrically with solutions of bixin in benzene of various known concentrations. By carrying out such a series of manipulations, the curve given in Fig 2 was obtained. For the sake of convenience and accuracy, the curve has been divided into three sections, each section giving the equivalence between solutions of stated concentrations. A formula anal-

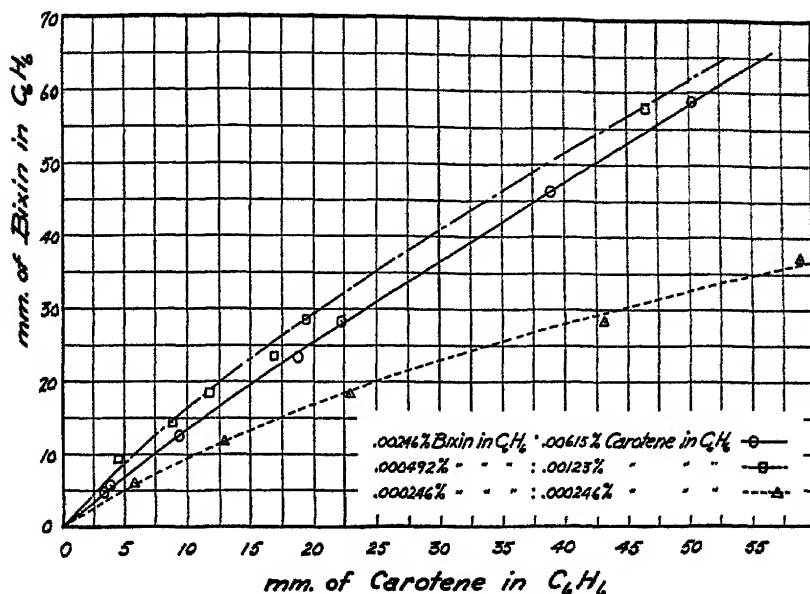


FIG 2 Colorimetric comparison of definite concentrations of carotene in benzene with definite concentrations of bixin in benzene

ogous to the one used for carotene in petroleum ether was used in determining the concentration of the unknown carotene solutions.

For example, suppose that a solution of carotene in benzene, of unknown concentration, is found to be approximately comparable to a 0.00246 per cent solution of bixin in benzene as far as depth of color is concerned. An exact color match is then obtained by use of the colorimeter. Let us say in this instance that the color of a

19.9 mm. depth of the carotene solution (concentration unknown) matches exactly the color of a 23.4 mm. depth of the bixin solution. Since a 0.00246 per cent bixin solution has been matched with a 0.00615 per cent carotene solution in plotting the graph of Fig. 2, the corresponding figure 0.00615 per cent is used in the following formula adapted from Palmer.

$$\text{Per cent carotene in unknown solution} = 0.00615\% \times \frac{\text{depth of carotene solution equivalent to standard}}{\text{depth of unknown carotene solution}}$$

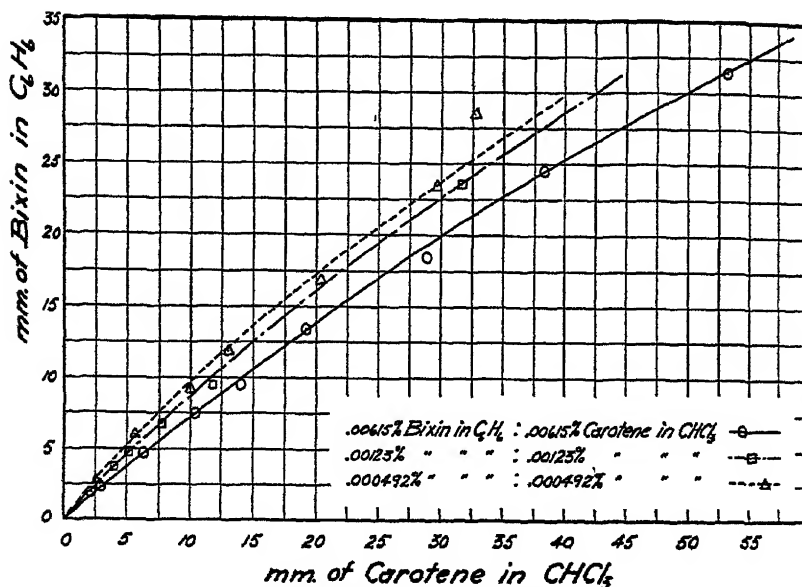


FIG 3 Colorimetric comparison of definite concentrations of carotene in chloroform with definite concentrations of bixin in benzene.

As read from the graph the depth of the carotene solution that is equivalent to the standard is 18.7 mm. Therefore, the concentration of the unknown solution is

$$\text{Per cent carotene} = 0.00615\% \times (18.7 \text{ mm} / 19.9 \text{ mm.}) = 0.00578\%$$

*Determination of Carotene in Chloroform*—Because of the optical difficulties encountered and already discussed above, it was apparent that dichromate solutions could not be used satisfactorily as standards for solutions of carotene in chloroform. It was thought

that bixin in chloroform might yield a good standard, but on trying to gather data for a complete graph, it was found that relatively concentrated solutions of bixin in benzene, however, gave excellent color matches at all concentrations that were of any value.

A solution of carotene in chloroform of known concentration was made in the manner indicated above and matched at different concentrations with a standard solution of bixin in benzene. The resulting curve is given in Fig. 3. Concentrations of unknown solutions of carotene were calculated as in the previous illustrations.

For example, a solution of carotene in chloroform, of unknown concentration, is found to be approximately comparable to a 0.00123 per cent solution of bixin in benzene as far as depth of color is concerned. An exact color match is then obtained by use of the colorimeter. Let us say in this instance that the color of an 18.4 mm. depth of carotene solution (concentration unknown) matches exactly the color of a 23.4 mm. depth of bixin solution. Since a 0.00123 per cent bixin solution has been matched with a 0.00123 per cent carotene solution in plotting the graph (Fig. 3), the figure 0.00123 per cent is used in the following formula.

$$\text{Per cent carotene in unknown solution} = 0.00123\% \times \frac{\text{depth of carotene solution equivalent to standard}}{\text{depth of unknown carotene solution}}$$

As read from the graph, the depth of carotene solution that is equivalent to the standard is 31.6 mm. Therefore the concentration of the unknown solution is

$$\text{Per cent carotene} = 0.00123\% \times (31.6 \text{ mm} / 18.4 \text{ mm.}) = 0.00211\%$$

#### SUMMARY

An accurate and convenient method for the colorimetric determination of carotene in such organic solvents as benzene and chloroform, where the Willstätter-Stoll method fails, has been devised.

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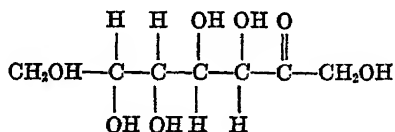
## THE UTILIZATION OF *d*-MANNOHEPTULOSE (*d*-MANNO-KETOHEPTULOSE) BY ADULT RABBITS

BY JOSEPH H. ROE AND C. S. HUDSON

(From the Department of Biochemistry, School of Medicine, George Washington University, and the National Institute of Health, United States Public Health Service, Washington)

(Received for publication, September 17, 1935)

*d*-Mannoheptulose, a 7-carbon ketose, was isolated from the fruit of the avocado tree, *Persea gratissima*, by La Forge (1) in 1917. La Forge found the configuration of this sugar is as follows:



This ketoheptulose occurs in the free state in the avocado fruit. The fact that this fruit has been regarded for centuries as an esteemed food made it seem probable that this sugar is assimilated by animals. Such a finding, if demonstrated, would be interesting because no 7-carbon monosaccharide has so far been observed to be utilized by the animal organism. A study of the physiological availability and metabolism of this sugar has therefore been undertaken.

### *d*-Mannoheptulose Tolerance

*Preparation of Mannoheptulose*—The mannoheptulose used in the experiments reported in this paper was prepared from avocado pears by Miss Edna Montgomery at the National Institute of Health following the method of La Forge (1). The product was of the highest purity, being free from perseitol and *l*-arabinose, and having an  $[\alpha]_D^{20}$  value of  $+29.3^\circ$  in water and a melting point of  $152^\circ$ .



*Method for Determination of Mannoheptulose in Blood*

Mannoheptulose reduces alkaline copper solutions and is not fermented by bakers' yeast. These properties were made the basis of a specific method for the determination of mannoheptulose in blood. In this procedure the blood is deproteinized by the  $\text{Zn}(\text{OH})_2$  method of Somogyi (2) and the filtrate is treated with thoroughly washed Fleischmann's yeast. The residual reducing substance after yeast fermentation is then determined by the copper reduction method of Benedict (3), a solution of mannoheptulose dissolved in saturated benzoic acid being used as a standard. Tests in which mannoheptulose was added to dog blood gave a 107 per cent recovery, which is very satisfactory in view of the fact that the Benedict procedure gives a slight blank with  $\text{Zn}(\text{OH})_2$  blood filtrates.

*Procedure*

Our first studies were of the nature of carbohydrate tolerance tests. Rabbits were fasted for 24 hours. After the urine was expressed from the animal's bladder and a control sample of blood was collected from the marginal ear vein, mannoheptulose was administered by mouth in some experiments and intraperitoneally in others. Samples of blood were then collected at hourly intervals for 4 hours, and the urine for 24 hours after administration was collected under toluene. The bloods were analyzed for total sugar by the Benedict method (3) and for mannoheptulose by the procedure described above. The total reducing substance in the 24 hour sample of urine was determined by the Shaffer-Somogyi method (4), with the latter authors' Reagent 50 with 5 gm. of KI.

*Results of Tolerance Studies*

The results of these experiments are shown in Table I. Following the administration of mannoheptulose, some mannoheptulose appeared in the blood as such and there was a marked increase in the total blood sugar. The concentrations of mannoheptulose in the blood were comparatively small, except in the experiment with Rabbit 5, in which a very large dose of the sugar was administered intraperitoneally. The mannoheptulose also stayed at a fairly constant level, while the total blood sugar showed a gradual increase, the last sample of blood collected having the highest value.

The mannoheptulose appearing in the blood will not account for the increments in total blood sugar; hence, the increases in total blood sugar are increases in reducing substance which is fermentable with yeast. These data therefore show that mannoheptulose undergoes a metabolic transformation in the rabbit in which fermentable reducing substance is formed.

That mannoheptulose was excreted in the urine in the experiments of Table I was demonstrated by boiling the urine with HCl

TABLE I  
*Data Showing Mannoheptulose Tolerance of Normal Rabbits*

Rabbit No	Weight	Method of administration	Dose	Blood sugar, mg. per 100 cc as glucose						Urine sugar Reducing substance in urine collected for 24 hrs after admin- istration, as mannoheptulose	
				Determination	Hrs after administration					Excretion	
					0	1	2	3	4		
	kg		gm per kg							gm	per cent
1	2 37	By mouth	2	Total sugar	91	116	126	132			
				Mannoheptulose		19	21	20		0 312	6.5
2	2.50	" "	5	Total sugar	92	115	125	131	135		
				Mannoheptulose		22	21	21	24	0 750	6 0
3	2 39	" "	5	Total sugar	60	100	115	121	126		
				Mannoheptulose		17	16	17	17	0 267	2 2
4	2 52	Intraperi- toneally	2	Total sugar	68	80	89	92	91		
				Mannoheptulose		15	14	14	15	0 315	6 2
5	2 32	"	5	Total sugar	101	428	485	502	509		
				Mannoheptulose		212	212	207	204	4 588	39 5

and testing for furfural, a reaction for mannoheptulose to which La Forge (1) called attention. The true mannoheptulose excretion was actually less than that indicated by the values shown in Table I, however, since there is some reducing carbohydrate in normal rabbit urine and the Shaffer-Hartmann reagent is sensitive to non-sugar reducing moieties of urine.

The urinary excretion of mannoheptulose in these experiments was small, considering the dosages used. In four of the experiments the total reducing substance in the urine ranged from 2.2 to

6.5 per cent of the mannoheptulose administered. In one experiment the urine for a second 24 hours after mannoheptulose administration was examined and did not show any more reducing substance than normal rabbit urine, thus eliminating the possibility of a delayed excretion of sugar. In the experiment with Rabbit 5 there was considerable excretion of mannoheptulose (39.5 per cent), but this result was obtained by intraperitoneal injection of a large dose of the sugar, an exaggerated experimental procedure in which the animal's body was rapidly flooded with mannoheptulose. The two experiments in which doses of 5 gm. per kilo of body weight were administered by mouth and not over 6 per cent of the sugar was excreted in the urine are especially significant and show that the rabbit has a high tolerance for mannoheptulose.

The above data obtained from an examination of the blood and urine following mannoheptulose administration thus seem to indicate that mannoheptulose is well utilized by the rabbit.

#### *Rôle of Liver*

##### *Procedure*

An experimental procedure was developed to determine the concentration of sugar of the afferent and efferent blood to the liver after mannoheptulose administration. In the experiments of Table II, Rabbits 1 and 2 were given mannoheptulose in 10 per cent solution by mouth and approximately 1 hour after administration the animals were anesthetized with nembutal and small amounts of ether. A longitudinal slit was made in the rabbit's abdomen and samples of blood were collected simultaneously from the portal vein and from one of the hepatic veins. In the experiments with Rabbits 3 and 4, the animals were anesthetized similarly and, after the abdomen was opened, mannoheptulose solution was injected into the duodenum. The animal's abdomen was then closed by means of clamps and, 30 minutes after injection of the sugar, samples of blood were collected simultaneously from the portal vein and a hepatic vein. The samples of blood were analyzed for total sugar and for mannoheptulose.

##### *Results*

The data of Table II show a low concentration of mannoheptulose in the portal blood. This was due either to slow absorption

of this sugar from the intestinal tract, or to a metabolic conversion of mannoheptulose into fermentable reducing substance in passing through the walls of the intestine. As the total sugar determinations reveal that there was a considerable increase in the total sugar of the portal blood, which was fermentable, the latter postulation is strongly suggested. These results also are of interest in that they show an apparently negative part played by the liver. The differences in the concentrations of mannoheptulose of the simultaneously collected samples of portal and hepatic blood are within the limits of experimental error and therefore seem to indicate that the liver does not participate in the metabolic transformation of mannoheptulose in the rabbit.

TABLE II  
*Rôle of Liver in Mannoheptulose Metabolism*

The total sugar and mannoheptulose of afferent and efferent blood to the liver after administration into the alimentary tract of mannoheptulose are measured in mg per 100 cc as glucose

Rabbit No	Dose	Vein	Total sugar	Mannoheptulose	Change in mannoheptulose
	<i>gm</i>				
1	10	Portal	94	20	+5
		Hepatic	99	25	
2	12	Portal	130	16	0
		Hepatic	190	16	
3	5	Portal	178	26	0
		Hepatic	180	26	
4	12	Portal	178	27	-3
		Hepatic	199	24	

#### *Effect of Insulin*

Crystalline insulin, of a potency of 18 units per mg., dissolved in 0.01 N HCl was used in these experiments. 3 units of the solution used produced convulsions in fasted 2 to 2.5 kilo rabbits within 1½ to 2½ hours after subcutaneous injection.

Two rabbits, fasted for 24 hours, were given 3 units of insulin subcutaneously. When the animals had convulsions, a solution containing 10 gm of mannoheptulose was injected intraperitoneally. One animal survived 2 hours and the other one 3½ hours after the onset of convulsions. Samples of blood were collected from the heart immediately after the death of the animals and were

analyzed for total sugar and for mannoheptulose. In the blood of one rabbit the total sugar and mannoheptulose were 296 and 302 mg. per 100 cc., respectively; for the other rabbit the total sugar and mannoheptulose were 325 mg. per 100 cc. of blood. Thus the analyses of postmortem blood from these two animals showed mannoheptulose present in large amounts, but *complete absence of fermentable sugar*. In the experiment with Rabbit 5 of Table I, an exactly parallel procedure except that insulin was not administered, there were present in the blood around 200 mg. of fermentable sugar per 100 cc. at approximately the same time after intraperitoneal injection of mannoheptulose. *Thus, when insulin was not injected, mannoheptulose administration brought about a marked increase in the fermentable sugar of the blood; and, when insulin was injected, fermentable sugar completely disappeared from the blood after mannoheptulose administration.* These data reveal that mannoheptulose is not a direct physiological antagonist to insulin, as there was a high concentration of this sugar in the blood of the two rabbits at the time of death; and they appear to indicate that insulin accelerates the oxidation of the fermentable substance which results from the metabolic transformation of mannoheptulose.

Further experiments were performed which corroborate the above findings. Two rabbits of approximately the same size as the two in the above experiment were fasted for 24 hours. 3 units of insulin were injected into each rabbit and at the same time 5 gm. of mannoheptulose in 10 per cent solution were given by mouth and 5 gm. of the sugar in a solution of the same concentration were injected intraperitoneally. One rabbit had convulsions twice, but recovered completely and survived; the other rabbit survived without any convulsions. It thus appeared from these experiments that mannoheptulose gives some protection against toxic doses of insulin, and if given at the same time that the insulin is administered, it may completely protect the animal against a minimum lethal dose of insulin.

In another experiment a rabbit was given 6 units of insulin and 10 gm. of mannoheptulose by mouth at the same time. This rabbit did not survive. In this experiment the rate of mannoheptulose conversion into the protective metabolite was not rapid enough to protect against a large dose of insulin.

## DISCUSSION

The data of this report show that mannoheptulose, when administered to rabbits, gives rise to the formation of a yeast-fermentable, copper-reducing substance in the blood of the animals. The removal of this substance from the blood is stimulated by insulin. The identity of this metabolite is obviously of considerable interest. In view of its behavior with yeast, with alkaline copper solution, and with insulin, properties corresponding to those exhibited by glucose and fructose, an interesting physiological finding, the metabolic transformation of a 7-carbon sugar into a sugar of lower carbon content, seems a possibility. Further work is planned to determine the identity of this substance.

## SUMMARY

1. Data have been obtained which show that mannoheptulose is physiologically available to the rabbit and that rabbits have a high tolerance for this sugar
2. In the rabbit mannoheptulose is converted into a yeast-fermentable, copper-reducing metabolite. The removal of this substance from the blood is stimulated by insulin.

The authors express their thanks to Miss Edna Montgomery and Dr. R. A. Hann, of the National Institute of Health, for valuable assistance.

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## THE QUESTION OF THE UTILIZATION OF TRYPTOPHANE ADMINISTERED SUBCUTANEOUSLY

BY VINCENT DU VIGNEAUD, ROBERT RIDGELY SEALOCK,  
AND CECIL VAN ETEN

*(From the Department of Biochemistry, School of Medicine, George  
Washington University, Washington)*

(Received for publication, September 17, 1935)

In a very interesting study of certain phases of tryptophane metabolism Alcock (1) arrived at a new conception of protein metabolism radically different from that generally accepted. A crucial point in the basis of this new theory was the failure to obtain growth of animals injected with tryptophane on a tryptophane-deficient diet. Alcock suggested that the limiting factor to growth on a tryptophane-deficient diet does not arise from the demand of the tissues for amino acids to be used for protein synthesis but that some material essential to life was made in the liver from tryptophane absorbed from the intestinal tract, whereas tryptophane delivered elsewhere than in the portal vein was not available for the "essential" purpose. He also concluded that the animal can synthesize its proteins while receiving no tryptophane and that for this particular purpose the body can make its own tryptophane. He further pointed out that this conclusion implied that the synthesis of a protein probably starts at a lower stage than the fully formed amino acid and that the amino acid groups actually found in the proteins have been formed there, not assimilated from the blood stream as such.

The experimental findings with respect to the utilization of injected tryptophane were of considerable interest to us as they were in direct contradiction to the experimental results we had previously obtained in our studies of the utilization of *d*- and *l*-tryptophane (2). We found that the body could utilize injected tryptophane for growth purposes.

Although we could find no instances in our previous work wherein we could suspect the presence of any artifacts, we felt



obligated to repeat our previous experiments in view of the negative experiments of Alcock and in view of the far-reaching conclusions on protein metabolism, mentioned above, that could be drawn from a non-utilization of injected tryptophane. However, repetition of our experiments led to exactly the same results

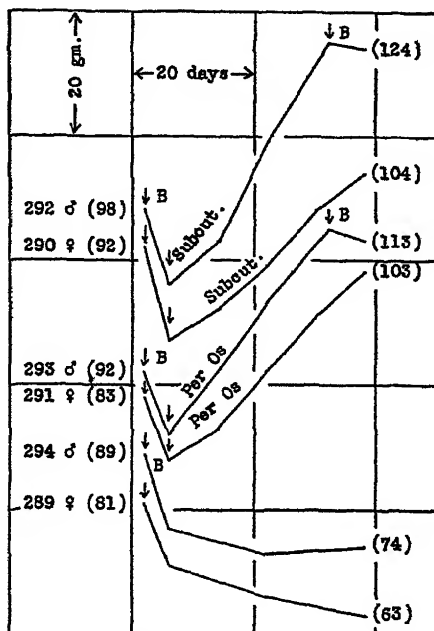


CHART I. Hydrolyzed casein diet. *B* represents the basal diet without any supplement. The arrow denotes the point of change. The number and sex of the rat are shown at the extreme left. Figures in parentheses indicate initial and final weights. Rats 293 and 291 received 5 mg. of tryptophane twice each day, and Rats 292 and 290 were given 2.5 mg. of tryptophane four times per day.

as we had previously reported. The growth curves shown in Chart I indicate clearly that the animals receiving injected tryptophane grew as well as those receiving it by mouth.

In our experiments we used a diet containing 14.7 per cent hydrolyzed casein free of tryptophane, whereas Alcock had used in his experiments a diet containing 20 per cent of zein supple-

mented with 5 per cent hydrolyzed casein. Thinking that the difference in basal diet might conceivably explain our opposite findings, we attempted to duplicate Alcock's diet as closely as possible. In these experiments we again obtained unquestionable utilization of injected tryptophane, as shown in Chart II.

In our original paper we expressly stated that the tryptophane was injected four times daily, whereas Alcock injected the trypto-

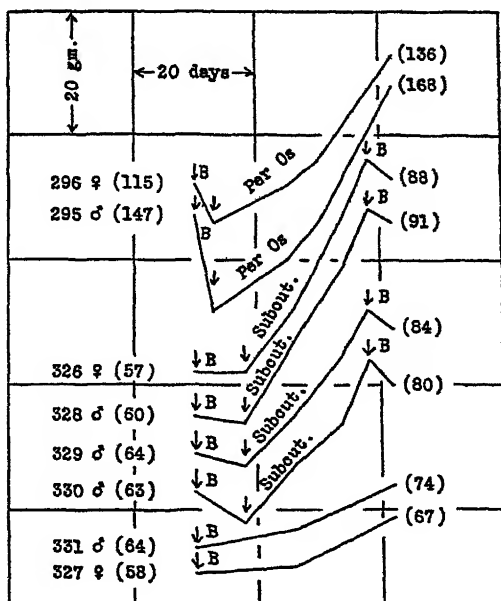


CHART II. Zein diet. Rats 296 and 295 received 5 mg. of tryptophane twice each day; Rats 326 and 328, 5 mg. of tryptophane four times per day; and Rats 329 and 330, 20 mg. once each day.

phane in one injection. As quoted by Alcock, Berg and Rose (3) showed that feeding 20 mg. of tryptophane per rat at 24 hour intervals gave submaximum growth, while 10 mg. doses at 12 hour intervals gave maximum growth. It might be thought that division of dosage would be even more significant in the case of injected material. In order to test, therefore, whether the number of injections per day might be the explanation of the difference between our results and Alcock's, we carried out an experiment

varying the number of injections per day. As will be seen in Chart II, utilization of the tryptophane was more efficient when the daily dose was administered in four portions at intervals during the day than when administered in one dose per day. But even in the latter instance we found a considerable difference in

TABLE I  
*Food Consumption*

Rat No and sex	Days	Average daily food consumption	Supplement	Rat No and sex	Days	Average daily food consumption	Supplement
		gm	mg per day			gm	mg per day
289 ♀	1-36	3 8		326 ♀	1- 8	3 5	
290 ♀	1- 4	7 8			9-28	6 1	20 (Subcutaneous)
	5-36	6 8	10 (Subcutaneous)		29-32	5 5	
291 ♀	1- 4	5 2		327 ♀	1-32	3 9	
	5-30	7 6	10 ( <i>Per os</i> )	328 ♂	1- 8	3 3	
292 ♂	1- 4	6 0			9-28	5 2	20 (Subcutaneous)
	5-30	8 4	10 (Subcutaneous)		29-32	4 5	
	31-36	5 3		329 ♂	1- 8	3 4	
293 ♂	1- 4	5 2			9-28	4 5	20 (Subcutaneous)
	5-30	6 1	10 ( <i>Per os</i> )		29-32	4 5	
	31-36	4 6		330 ♂	1- 8	3 9	
294 ♂	1-36	4 3			9-28	4 3	20 (Subcutaneous)
295 ♂	1- 3	5 3			29-32	3 8	
	4-32	9 1	10 ( <i>Per os</i> )	331 ♂	1-32	4 3	
296 ♀	1- 3	6 3					
	4-32	7 2	10 ( <i>Per os</i> )				

the rate of growth of the injected animals and of the controls on the basal diet. The food consumption in all these experiments is given in Table I.

#### EXPERIMENTAL

In duplicating the results of our earlier experiments in which the tryptophane was administered subcutaneously, the same experimental conditions as previously described were used. Six albino rats were put on a basal diet deficient in tryptophane. This consisted of acid-hydrolyzed casein 14.7, cystine 0.3, dextrin

40, sucrose 15, lard 17, cod liver oil 5, salt mixture (Osborne and Mendel (4)) 4, and agar 2 per cent. The diet was furnished *ad libitum*, and yeast pills consisting of 150 mg. of brewers' Yeast Vitamine-Harris and 75 mg of dextrin were fed at 12 hour intervals. At the end of the first 4 days, when all the animals had lost weight on the basal diet, two of them were given tryptophane by mouth. 5 mg. of the tryptophane were incorporated in the yeast pills and one pill was fed to each of the two animals at 12 hour intervals. Two other animals were given 10 mg. of tryptophane daily by subcutaneous injections. The tryptophane was dissolved in water to make a 1 per cent solution and 0.25 cc. was injected at 9 a.m., 1 p.m., 6 p.m., and 10 p.m. The remaining two animals were left on the basal diet as controls. As shown in Chart I, the animals receiving tryptophane subcutaneously grew at the same rate as those receiving tryptophane by mouth.

In the experiments with the protein employed by Alcock, the basal diet was composed of acid-hydrolyzed casein 5, zein 20, dextrin 40, sucrose 20, lard 10, and salt mixture 5 per cent, and differed from Alcock's only in that we substituted lard for palm kernel oil and dextrin for rice starch. The zein was prepared from corn gluten by the customary alcoholic extraction method. For this group of experiments, eight animals were used. This diet was also furnished *ad libitum*. To supply the necessary vitamins, 1 drop of cod liver oil was put on the food each day and yeast was given in pills as described above. Two of the animals received 5 mg. of tryptophane incorporated in the yeast pills, as mentioned above, twice a day by mouth. A second pair received the tryptophane supplement by subcutaneous injection of 1 cc. of a 2 per cent solution, or 20 mg. daily. To two others, tryptophane was administered subcutaneously in four equal doses daily, each dose consisting of 5 mg. in a 2 per cent solution. The remaining two animals served as controls, receiving no added tryptophane. The growth curves are shown in Chart II and the food consumption is recorded in Table I.

#### SUMMARY

It has been clearly demonstrated that tryptophane injected subcutaneously can be utilized, in contrast to the results reported by Alcock and in confirmation of our previously reported results.

From our findings we cannot feel that there is experimental

evidence to support the theories of protein metabolism promulgated by Alcock, based on the non-utilization of injected tryptophane.

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## STUDY OF THE SERUM LIPIDS BY A MICROGRAVIMETRIC TECHNIQUE

BY WILLIAM R. WILSON AND ARILD E. HANSEN\*

(From the Department of Pediatrics of Yale University School of Medicine  
and the Pediatric Service of the New Haven Hospital, New Haven)

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The purpose of this publication is to present a method for the separation of the saponifiable and unsaponifiable fractions of the blood lipids and the quantitative determinations of these fractions by a microgravimetric technique. The method of separation of these fractions is an adaptation of one used by Bloor (1) in an early study, while the gravimetric technique is similar to that used by Wilson and Hanner (2) for the total lipids. The alcohol-ether extract of 1 cc. of serum is saponified with 50 per cent KOH, evaporated to dryness, and, after adding water and acidifying, extracted with petroleum ether. A phosphorus determination is made on the watery residue after removal of the petroleum ether extract. The saponifiable material is removed from the petroleum ether extract by treating with alcoholic KOH solution and water. The petroleum ether, which contains only the unsaponifiable fraction, forms an upper layer that is quantitatively removed for weighing. The fatty acids are recovered from the alkaline alcohol-water solution by evaporating to dryness, dissolving in water, precipitating with acid, and extracting with petroleum ether. The petroleum ether extract containing the fatty acids is quantitatively removed for weighing. The acidimetric titration value and iodine number are then determined on the weighed fatty acid fraction.

### *Method*

#### *Reagents*

1. Alcohol-ether (3:1). Bloor's mixture of 3 parts of redistilled ethyl alcohol, 95 per cent (refluxed 3 hours with NaOH sticks and

\* Alexander Brown Coxe Fellow, 1934-35.

redistilled), and 1 part of ethyl ether, redistilled or anesthetic quality.

2. 50 per cent KOH. 50 gm. of KOH dissolved in 100 cc. of distilled water.

3. Hydroquinone, 0.1 per cent solution in 95 per cent alcohol. This should be kept in a dark place and discarded when a pinkish hue appears

4. Concentrated HCl. Sp. gr about 1.19.

5. 0.1 N KOH in redistilled 95 per cent ethyl alcohol.

6. Petroleum ether, b.p. 30–58°. This consists largely of pentane and should leave no weighable residue upon the evaporation of 50 cc. of the material.

7. 2 per cent phenolphthalein in 95 per cent ethyl alcohol.

8. 0.02 N NaOH.

### *Technique*

*Preparation of Extract, Containing Total Lipids*—Prepare the alcohol-ether extract in the usual manner, 2.5 to 5 cc. of serum or plasma being used, and reflux for 1 hour on the steam bath or hot-plate (3). Filter into a 100 cc. volumetric flask, washing the precipitate three or four times with alcohol-ether (3:1) mixture, and bring to volume at room temperature

*Saponification and Extraction of Total Lipids*—Measure duplicate aliquots (equivalent to approximately 1 cc. of serum) of the alcohol-ether extract into 125 cc. Erlenmeyer flasks. Add 0.1 cc. of 50 per cent KOH, and 0.1 cc. of 0.1 per cent alcoholic solution of hydroquinone. Saponify by heating for 1 hour at about 80°. When the material is almost dry, but still slightly pasty, remove the last traces of alcohol by applying light suction to the flask. Add 3 to 5 cc. of distilled water to dissolve all the soaps. Add 0.1 cc. of concentrated HCl, drop by drop, and agitate until the particles clump. Add 10 to 15 cc. of petroleum ether. Stopper the flasks and allow them to stand for about 3 hours or place them in the shaking machine at low speed for about 20 minutes. Transfer the clear supernatant petroleum ether layer to a 50 cc. round bottomed centrifuge tube by means of a small rubber bulb pipette with a capillary tip. Complete the transfer of the lipid material by successively adding and transferring several 5 to 8 cc. portions of petroleum ether.

*Determination of Phosphorus Derived from Saponifiable Phospholipids*—The residue after the extraction with petroleum ether may be discarded. To determine the lipid phosphorus liberated during the saponification, transfer the residue to a 50 cc. volumetric flask with successive portions of alcohol and water and bring to volume at room temperature. Take 10 cc. aliquots (equivalent to 0.2 cc. of serum) and determine the phosphorus by the same technique used for lipid phosphorus.

*Calculations*—These are the same as for lipid phosphorus.

*Separation of Unsaponifiable Fraction*—Evaporate the petroleum ether extract down to a volume of about 5 cc. by placing the tubes in front of an electric fan or in a water bath at 65°. Add 5 cc. of 0.1 N KOH in alcohol, washing down the sides of the tube. Then add 5 cc. of distilled water. Emulsify the mixture with a glass rod. When thoroughly mixed, allow to stand a few minutes, and emulsify again. Wash the glass rod with 1 to 2 cc. of distilled water, followed by 2 to 3 cc. of petroleum ether. Centrifuge or allow to stand until both layers are clear. Transfer the supernatant fluid by means of a rubber bulb pipette to a tared weighing bottle of 15 to 20 cc. capacity. Complete the transfer by washing three to four times with successive 2 to 3 cc. portions of petroleum ether. Evaporate the contents of the weighing bottle to dryness and allow to come to constant weight by standing several hours in the balance room. The weight obtained is that of the unsaponifiable fraction.<sup>1</sup>

*Calculation*

$$\frac{\text{Mg. unsaponifiable material} \times 100}{\text{Cc serum in sample}} = \frac{\text{mg. unsaponifiable material per}}{100 \text{ cc. serum}}$$

*Iodine Number of Unsaponifiable Fraction*—For the determination of the iodine number, transfer the contents of the weighing bottle to a 125 cc. glass-stoppered iodine flask with two successive 1 cc. portions of chloroform.

<sup>1</sup> On account of the slight change in tare weights, it was necessary to make a control weighing of a weighing bottle of similar size with each set of determinations. This was necessary in spite of the fact that the temperature in the weighing room did not vary more than 6°F. The inconstancy of the weights apparently is accounted for by the variations in humidity. The Pregl type of balance was used, but a chainomatic balance sensitive to 0.05 mg. was found to be fairly satisfactory in a few parallel weighings.



The Rosenmund-Kuhnhehn method as described by Yasuda (4), was found to be satisfactory for this determination

*Calculation*

$$\frac{\text{Cc. } 0.01 \text{ N Na}_2\text{S}_2\text{O}_3 \times 127}{\text{Weight of sample in mg.}} = \text{I No. of unsaponifiable fraction}$$

*Recovery of Saponifiable Fraction*—To the saponifiable fraction, contained in the 50 cc. centrifuge tube, add 0.1 cc. of 0.1 per cent hydroquinone solution, place in a beaker of warm water, and evaporate just to the point of dryness or to a slightly pasty consistency. Suction may be applied to remove the last traces of alcohol. Add 5 cc. of distilled water, to dissolve the soaps. Acidify with 0.07 to 0.08 cc. (2 drops) of concentrated HCl, and agitate until clumping occurs. Add 8 to 12 cc. of petroleum ether, and centrifuge or allow to stand until both layers are clear. Transfer the supernatant layer to a tared weighing flask (about 20 cc. capacity), using the rubber bulb pipette. Complete the transfer with four to five successive washings with 2 to 3 cc. of petroleum ether.

Add exactly 0.1 cc. of 0.1 per cent hydroquinone solution to the material in the weighing bottle. After the material is evaporated to dryness, the bottles are weighed. Deduct 0.10 mg. from the weight obtained, to correct for the hydroquinone. To correct for changes in the tare a control weighing is made here as described.<sup>1</sup> The weight obtained is that of the saponifiable or fatty acid fraction.

*Calculation*

$$\frac{\text{Mg saponifiable material} \times 100}{\text{Cc. serum used}} = \text{mg. saponifiable material per 100 cc. serum}$$

*Acidimetric Titration of Fatty Acids and Calculation of Their Average Molecular Weight*—After the weight has been obtained, add 5 cc. of 95 per cent alcohol to the sample and warm. Add 1 drop of 2 per cent phenolphthalein and titrate while still warm with 0.02 N NaOH to the first pink color that persists. Run a

blank titration on hot alcohol and phenolphthalein and subtract this from the titration figure for fatty acids.

*Calculation*

$$\frac{\text{Cc. } 0.02 \text{ N alkali} \times 20}{\text{Cc. serum used}} = \text{milli-equivalents of fatty acid per liter}$$

$$\frac{\text{Mg. in saponifiable fraction} \times 1000}{20 \times \text{cc. } 0.02 \text{ N alkali}} = \text{average molecular weight of fatty acids}$$

*Saponification and Recovery of Fatty Acids for Determination of Iodine Number*—After the titration add 2 drops of 50 per cent KOH to increase the solubility of the soaps, followed by 0.1 per cent hydroquinone,<sup>2</sup> and transfer to a 125 cc. Erlenmeyer flask. Complete the transfer with three successive 1 cc. portions of alcohol. Evaporate to dryness. Add 2 to 5 cc. of distilled water, to dissolve the soaps, then add 2 drops (about 0.07 to 0.08 cc.) of concentrated HCl. Agitate gently for a few moments until clumping of the fatty acids occurs. Extract with petroleum ether and transfer the fatty acids to a 125 cc. glass-stoppered Erlenmeyer flask and evaporate to dryness by the techniques previously described. Dissolve the fatty acids in 2 cc. of chloroform for the determination of the iodine number. Yasuda's (4) technique was found to be satisfactory. Run a blank determination on 30 cc. of petroleum ether, evaporated to dryness, as it may be necessary to make a slight correction for the petroleum ether.

*Calculation*

$$\frac{\text{Cc. } 0.01 \text{ N Na}_2\text{S}_2\text{O}_3 \times 127}{\text{Mg. saponifiable material}} = \text{average I No of saponifiable fraction}$$

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<sup>2</sup> The danger of oxidation of the fatty acids, especially when heat is applied, is well recognized. There is considerable evidence that hydroquinone in small quantities prevents changes in iodine number due to oxidation. The hydroquinone should not be present during the bromine reaction as it takes up a small amount of bromine. Hydroquinone is not extracted from an aqueous solution by petroleum ether. Old hydroquinone may contain oxyquinone, which has an iodine number of 470, and if present may cause considerable error. No hydroquinone is present during the actual determination of the iodine number.

*Lipoid Phosphorus*—Use aliquots equivalent to 0.2 cc. of serum for the determination of the lipoid phosphorus. The material first is oxidized following Baumann's (5) procedure and the inorganic phosphate is then determined by any standard colorimetric method, such as that of Benedict and Theis (6).

*Calculation*

$$\frac{5R}{\text{Cc. serum used}} \times \text{mg. P in standard} = \text{mg lipoid P per 100 cc. serum}$$

*R* is the reading of the standard with the unknown set at 20.

#### DISCUSSION

The alcohol-ether extract of the serum contains all of the lipids and, in addition, small amounts of glucose, glutathione, some inorganic salts, and still smaller quantities of other substances. Repeated determinations have shown that inorganic phosphorus is not present in quantity sufficient to produce a color reaction. After the saponification, acidification, and extraction, the total lipid may be divided into three fractions, saponifiable, unsaponifiable, and water-soluble residue after saponification. Extraction with petroleum ether removes the saponifiable and unsaponifiable fractions unaccompanied by appreciable quantities of the other substances mentioned.

In order to estimate the total lipid in the blood the amounts of water-soluble derivatives must be added to the sum of the saponifiable and unsaponifiable fraction. This fraction consists of glycerol and the non-fatty acid portion of the phospholipid. The glycerol content may be calculated from the number of milli-equivalents of fatty acid per liter, on the basis of a molecular weight of 41 for the trivalent  $\text{C}_3\text{H}_5$ , which is the portion of the glycerol represented in neutral fat and the portion of the phospholipid bound to fatty acid. The formula used in this calculation is  $1.37 \times \text{milli-equivalents of fatty acid} = \text{mg. of glycerol per 100 cc.}$  The remaining phospholipid residue may be calculated from the phosphorus content of the residue after saponification. The empirical formula for that portion is  $\text{CH}_2\text{—O—HPO}_3\text{—C}_5\text{H}_{13}\text{N}$  (7), with a molecular weight of 181, about 6 times the molecular weight of phosphorus. Therefore, the mg. per cent of the water-soluble residue is estimated to

be  $(1.37 \times \text{milli-equivalents of fatty acid}) + (6 \times \text{mg. per cent of P in watery residue})$ .

Comparison was made in seven instances between the total lipid as determined by the gravimetric method of Wilson and Hanner (2), in which there is no saponification, and the sum of the fractions determined by the present method. The material unaccounted for averaged 3.8 per cent of the total lipid, the sum of the three fractions being less than the total in every instance.

The method suggested by Bloor apparently makes a satisfactory separation of the two fractions as indicated by the results obtained in an analysis of known solutions. On account of the difficulty of obtaining a satisfactory sample of lecithin, the known solutions were made up to contain only fatty acids and cholesterol. Solutions containing mixtures of palmitic acid, linoleic acid, and cholesterol were used. Six different mixtures were analyzed in duplicate and values within 2 per cent of the theoretical were obtained for unsaponifiable material, saponifiable material, titration value, and iodine number. Some uncertainty was felt as to the possible effect of the presence of phospholipid on the separation, but it apparently does not interfere to any appreciable extent. The saponifiable fractions from three blood samples were tested for cholesterol by the Liebermann-Burchard color reaction and the color produced was not greater than that produced by a blank or by a small quantity of palmitic acid used as a control. Blanks run for comparison showed no weight or titration value, indicating the absence of inorganic acid or salt in the fatty acid fraction. Lipoid phosphorus was present in the saponifiable fraction to the extent of about 0.2 to 0.5 mg. per 100 cc. Lack of data as to the form in which the phosphorus occurs in the saponifiable fraction makes it difficult to allow for it in calculation of molecular weights and iodine numbers. Since substances other than fatty acids were not found in amounts greater than a maximum of 12.5 mg. per cent, we assume that the saponifiable fraction, as determined by the method presented, represents for practical purposes the isolated total fatty acids of the serum.

### Results

*Human Sera*—The values on a number of human sera are summarized in Table I. Inspection of these data reveal that they are



in essential agreement with the values given for the serum lipids by various workers (8).

*Unsaponifiable Fraction*—The unsaponifiable fraction contains cholesterol, unsaponifiable phospholipid, and small amounts of pigment. In two instances the determination of cholesterol by the digitonin method showed that about 70 per cent of the material in this fraction is cholesterol. Phosphorus determinations indicate that almost all of the remainder is unsaponifiable phospholipid. Tests for inorganic phosphorus gave no color, indicating that the phosphorus is present in organic form. The slight discrepancy between the total unsaponifiable and the sum of the cholesterol and unsaponifiable phospholipid (about 10 mg per cent) is within the limits of error of the methods employed, but it does not exclude the possibility that there may be some unidentified material present. The iodine numbers of the unsaponifiable fraction range from 59.2 to 66.1 with an average of 63.4. Cholesterol has a theoretical iodine number of 66. However, by the pyridine-sulfate-dibromide method, Yasuda (4) obtained values of 69 for cholesterol. The values obtained indicate that some material besides cholesterol may absorb a small quantity of iodine.

*Saponifiable or Fatty Acid Fraction*—The saponifiable fraction is composed of a mixture of fatty acids derived from three chief sources—triglycerides, cholesterol esters, and phospholipids. Bloor (1) and Channon and Collinson (9) have shown that the saponifiable fraction consists of a mixture of fatty acids with widely different characteristics. The average molecular weight and average iodine number give an indication of the predominating components of this mixture.

The average molecular weight obtained in this study was 291 (Table I). If the maximum allowance is made for the presence of non-fatty acid material in the saponifiable fraction, then the average molecular weight would be 281. These figures indicate the predominance of  $C_{18}$  fatty acids. It is usually stated that the blood contains some of the shorter chain fatty acids, especially those of the  $C_{16}$  series. The average of 280 to 284 would indicate that there must be an appreciable quantity of the longer chain ( $C_{20}$ ,  $C_{22}$ , or  $C_{24}$ ) acids in order to give such a high average molecular weight in the presence of the shorter chain acids. Channon and Collinson (9), in studies of the lipids in ox blood, found that these

longer chain fatty acids are present in appreciable quantities. Tangl (10) found this to be true for dog, ox, and rabbit blood, while Brown (11) has found the same in human serum. The average molecular weights of the fatty acids obtained by Channon and Collinson who use large quantities of ox blood are in close agreement with our values for human serum. In one large (40 liter) sample the average molecular weight was 294.

The study of these workers (9) indicates that very little (about 15 mg. per cent) of the fatty acids in ox blood is present as the triglyceride. Since free fatty acid is probably not present in serum, the triglycerides may be calculated by subtracting from the total fatty acids the sum of those combined to phospholipid and cholesterol. For this calculation, it was assumed that 60 per cent of the total cholesterol was combined with fatty acids. The sum of the fatty acids from these two sources amounted to a little more than half of the total, indicating that in human serum a substantial part of the fatty acids are combined in the form of triglyceride.

The iodine numbers of the fatty acids are found to be between 99 and 113 (Table I). This is in substantial agreement with the values found by some workers (12, 13), and somewhat higher than those found by others (14, 15). It should be borne in mind that these numbers are calculated directly from the weight of the isolated fatty acids. If a correction were applied for the small amount of material that is not fatty acid, as stated in the discussion of molecular weights, then these values for the iodine number would be increased slightly.

The iodine number is really a measure of the number of double bonds present in a given amount of lipid material. Fatty acids with 14, 18, and 20 carbon atoms and one double bond have iodine numbers of 112, 90, and 82 respectively. For identification it seems preferable to express the iodine absorption capacity of fatty acids directly in terms of the number of double bonds per molecule. This may be readily calculated by the formula (molecular weight  $\times$  iodine number)/25,400. It may also be calculated by the formula ( $\text{mm I}_2 \div 2$ )/mm fatty acid.

In this study the fatty acids were found to have an average of 1.23 double bonds per molecule, the range being between 1.15 and 1.33. This indicates that the predominant fatty acids have one

double bond; but appreciable quantities of fatty acids having two or more double bonds are also present. If there is a considerable amount of saturated fatty acid in the mixture, in order to have an average of 1.23 double bonds per molecule, there must be a correspondingly greater amount of acid having two or more double bonds. The characteristics of the mixture (molecular weight 280 to 290 and 1.23 double bonds per molecule) more nearly resemble those of a mixture of oleic and linoleic, possibly with some stearic, than any of the other fatty acids. The presence of arachidonic acid ( $C_{20}H_{32}O_2$ ) in small quantities in the blood plasma has been demonstrated previously. It is probable, therefore, that the fatty acids of human serum are predominantly unsaturated acids with 18 carbon atoms, with some acids of 20 carbon atoms, and smaller amounts of other acids.

*Phospholipids*—The phospholipids of the blood behave in a manner indicating the presence of two or more substances of this character. It has been assumed by most workers that the phospholipid of the blood is largely in the form of lecithin. MacLean (7) points out that there may be numerous lecithins, characterized by the presence of different fatty acids in their make-up. No attempt has been made in the present study to determine definitely the character of the two fractions into which the phospholipid is divided following saponification. Calculations based on the amount of phosphorus in the saponified portion, however, indicate that the phospholipid saponified is probably a lecithin. With the amounts of blood used in this study, it could not be ascertained whether the unsaponifiable phospholipid is a lecithin, which is refractory to hydrolysis, or is one of the other phospholipids known to occur in the body. The unsaponifiable phospholipid amounts to from 7 to 32 per cent of the total and is presumably different in some way from the portion saponified.

#### SUMMARY

A method is presented for the separation and weighing of the unsaponifiable and saponifiable portion of the lipid in aliquots equivalent to 1 cc. of serum. The unsaponifiable fraction consists of cholesterol and the unsaponifiable phospholipid. The saponifiable portion consists of fatty acids with the possible admixture of small amounts of other substances. The iodine number of the



unsaponifiable portion and the iodine number and titration value of the fatty acids are determined. The glycerol, phosphoric acid, and choline resulting from the saponification of the neutral fat and phospholipid are left behind in the residue from the extraction with petroleum ether. Lipoid phosphorus has been determined on both the alcohol-ether extract and this residue.

Analyses of seventeen normal sera are presented with the following results.

1. The total lipid averages 657 mg per 100 cc.
2. The unsaponifiable fraction averages 260 mg. per 100 cc. with an iodine number of 63.4.
3. The saponifiable fraction averages 362 mg. per 100 cc. with an average molecular weight of 291 and an iodine number of 108, equivalent to 1.23 double bonds per molecule. These figures suggest that while 18-carbon unsaturated fatty acids predominate in human blood, there are appreciable quantities of longer chain unsaturated fatty acids.
4. The division of lipid phosphorus between the unsaponifiable fraction and the residue from the saponification indicates the presence of two types of phospholipid in the blood, one of them saponifiable and the other not saponifiable. The saponifiable fraction averages about 81 per cent of the total lipid phosphorus.

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## THE MICRODETERMINATION OF FERROCYANIDE IN MUSCLE AND URINE\*

By J. GRAHAM EDWARDS AND WILSON D. LANGLEY

*(From the Departments of Anatomy and Biological Chemistry, the University  
of Buffalo Medical School, Buffalo)*

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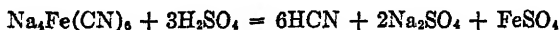
In the study of the function of the differentiated parts of the renal unit, numerous experiments have been performed involving the injection of various substances into the living animal in order to ascertain the localization of such function. Iron salts were among the substances so injected—especially sodium ferrocyanide. Because of the attempt of several investigators to locate histologically, within the unit, the site of the excretion of ferric iron or of iron cyanide complexes (1-8), and because of the close correlation between renal structure and its specific function, it seemed valuable to determine the content of ferrocyanide present in urine or tissue after its injection into normal and nephropathic animals. Since the animals experimented upon were often small and the amount injected proportionately so, it was necessary to devise a method for the determination of small amounts of ferrocyanide present in such complex solutions as urine or those obtainable from tissues.

Of the published methods for determining ferrocyanide quantitatively, none was found suitable for its determination in minute quantities in complex solutions, or for its removal from the various components of urine or tissue which might limit the amount recoverable and thus interfere with the determination. A means of avoiding possible interference of such components which the older method of Leschke (3) and the recent one of Stieglitz and Knight (9) do not obviate, was suggested by Williams' method

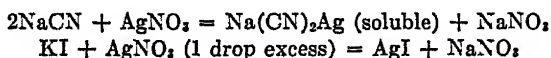
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(10). The latter involves the hydrolysis of the ferrocyanide with sulfuric acid and the determination of the liberated HCN. By so modifying this procedure as to make it quantitative, and by using an accurate titration of the  $\text{CN}^-$  with standard silver nitrate (11, 12), a method of analysis has been evolved for determining, with considerable accuracy, a few mg. of iron complex per 100 gm. of muscle or urine

The hydrolysis reaction is as follows:



The use of cuprous chloride, as suggested by Williams, is not necessary. With the proper concentration of sulfuric acid, all the  $\text{CN}^-$  is liberated as HCN. This is aerated into a solution of NaOH, in which it was held as NaCN. After the completion of the hydrolysis, the  $\text{CN}^-$  is titrated with  $\text{AgNO}_3$ , as follows:



The fact that in the hydrolysis 6 moles of HCN are produced per mole of complex increases 3-fold the accuracy of the determination of the complex.

The end of the reaction is indicated by the appearance of colloidal silver iodide, which gives an immediate turbidity. In order to see the Tyndall effect of AgI clearly, a beam of light is passed through the solution during the titration.

### *Apparatus*

Two vertical condensers are connected across the top, as in Fig. 1. Rubber stoppers are used. To the first is attached a modified 300 cc Kjeldahl flask (Fig. 1, 1), and to the second, as a receiver, a 200 × 25 mm. test-tube. The second condenser is packed loosely with glass wool and beads in order to increase its efficiency. Suction is so provided that a current of air can be washed through NaOH and then drawn through the Kjeldahl flask, both condensers, and the receiving tube

A microburette and a spot light are used for the titration.

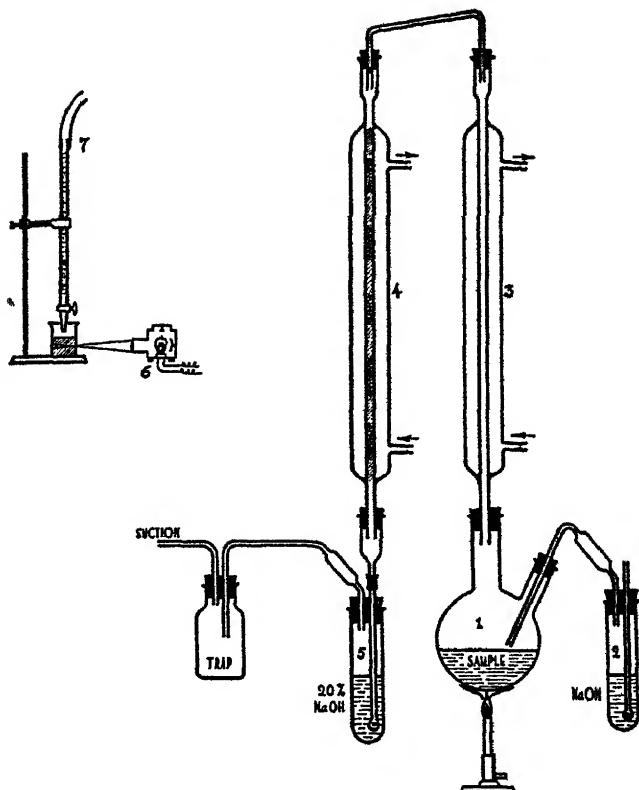


FIG 1. A diagram of the apparatus used in the microdeterminations of ferrocyanide. 1, Kjeldahl flask with side arm; 2, test-tube for washing air; 3, water-cooled, vertical reflux condenser; 4, the same as 3, but containing beads and glass wool in alternate layers, 5, test-tube as a receiver; 6, spot light; 7, microburette; between the lower end of 4 and the bubbling rod of 5, a piece of rubber tubing is preferably used.

### Reagents

$\text{AgNO}_3$ . 0.01 N, standardized against HCl of known normality, by the Volhard process.

$\text{Na}_4\text{Fe}(\text{CN})_6 \cdot 10\text{H}_2\text{O}$ . Analyzed by precipitation of the iron as the hydroxide, with subsequent ignition, and weighing of the oxide.

30 per cent  $\text{H}_2\text{SO}_4$ .

20 per cent NaOH.

10 per cent KI.

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*Procedure*

The receiver, containing 25 cc. of 20 per cent NaOH (Fig. 1, 5), is connected to the second condenser (Fig. 1, 4) and to the suction pump. The substance or solution to be analyzed is placed in the Kjeldahl flask, and enough water added to make a volume of about 100 cc., followed by the addition of 20 cc. of sulfuric acid, and then

TABLE I  
*Analysis of Sodium Ferrocyanide Solutions*

Na <sub>4</sub> Fe-(CN) <sub>6</sub> used	Water		Urine*		Muscle†	
	AgNO <sub>3</sub> ‡ required	Na <sub>4</sub> Fe(CN) <sub>6</sub> determined	AgNO <sub>3</sub> required	Na <sub>4</sub> Fe(CN) <sub>6</sub> determined	AgNO <sub>3</sub> required	Na <sub>4</sub> Fe(CN) <sub>6</sub> determined
mg	cc	mg	cc	mg	cc	mg
1	0 79	1 00	0 74	0 94		
	0 78	0 99	0 74	0 94		
2	1 62	2 06	1 54	1 96	1 40	1 78
	1 58	2 01	1 56	1 98	1 26	1 60
4	3 11	3 95	3 02	3 84	2 64	3 53
	3 08	3 92	3 10	3 94	2 58	3 45
10	7 82	9 95	7 62	9 70	7 30	9 29
	7 82	9 95	7 66	9 74	7 36	9 36
20	15 60	19 85	15 60	19 85	14 94	19 01
	15 58	19 82	15 38	19 57	14 89	18 95
40	30 40	38 68	30 74	39 12	31 00	39 45
	30 15	38 37	30 93	39 36	31 10	39 57

\* Urine of man was used

† 12 gm portions of minced muscle were thoroughly macerated with a concentrated solution of Na<sub>4</sub>Fe(CN)<sub>6</sub>, and the mixture was allowed to stand for at least 15 minutes. The time of distillation was 45 minutes with cold, and 15 minutes with hot condensers; 40 cc. of 30 per cent sulfuric acid were used. Decrease either in the amount of sulfuric acid, or in the time of distillation gave incomplete recovery of cyanide. Blanks for pure solutions and for urine were negligible. That for the muscle was 0 03 cc. of AgNO<sub>3</sub>. Corrections were not made.

‡ 1 cc. = 1 34 mg. of Na<sub>4</sub>Fe(CN)<sub>6</sub> · 10H<sub>2</sub>O.

a few quartz pebbles. The flask is immediately connected to the first condenser and to the air-washing bottle which contains 20 per cent NaOH. The contents of the flask are refluxed for 20 minutes (for muscle, 45 minutes), during which time the HCN is aerated through the cold condensers. The flow of water through the condenser is then stopped but the refluxing is continued until

the first condenser becomes hot. This usually requires 10 minutes (for muscle, 15 minutes). The condensers are then disconnected, the second one is washed, and the contents of the receiver are transferred to a beaker and titrated with 0.01 N  $\text{AgNO}_3$ , the microburette and spot light being used.

TABLE II

*Effect of Changing the Time of Distillation upon Amount of Ferrocyanide Recovered*

	$\text{Na}_4\text{Fe}(\text{CN})_6$ used	Heating time		$\text{Na}_4\text{Fe}(\text{CN})_6$ recovered	Heating time		$\text{Na}_4\text{Fe}(\text{CN})_6$ recovered
		Cold condenser	Hot condenser		Cold condenser	Hot condenser	
	mg	min	min	per cent	min	min	per cent
Water	8	10	10	98.1	20	10	99.2
	10	10	10	96.0	20	10	99.2
	20	10	10	97.0	20	10	99.2
	40	10	10	91.6	20	10	96.5
Muscle	10	20	10	82.2	45	15	93.3

TABLE III

*Effect of Changing Amount of Sulfuric Acid upon Amount of Ferrocyanide Recovered*

	30 per cent $\text{H}_2\text{SO}_4$ used	$\text{Na}_4\text{Fe}(\text{CN})_6$		30 per cent $\text{H}_2\text{SO}_4$ used	$\text{Na}_4\text{Fe}(\text{CN})_6$ recovered
		Used	Recovered		
	cc	mg	per cent	cc	per cent
Water	20	10	99.2	10	99.2
	20	20	99.6	20	100.1
	20	40	96.5	40	97.5

The distillation time was 20 minutes with cold and 10 minutes with hot condenser.

In Table I are shown figures which were obtained by analysis of solutions of  $\text{Na}_4\text{Fe}(\text{CN})_6$  in water, urine, and in minced muscle.

The recovery of ferrocyanide from muscle was less complete and more time-consuming than from simple solutions. In separate experiments in which chlorides, bromides, and iodides were added it was shown that halogen-cyanide complexes were not the cause. The accuracy of the determinations made upon water or urine

solutions was approximately 98 per cent for samples larger than 10 mg. In the determinations upon the smaller amounts of ferrocyanide no unusual precautions were taken. It is indicated that by reducing the size of the condensers and by standardizing the conditions more carefully, higher accuracy might easily be obtained.

After it was established that quantitative determinations of ferrocyanide could be made upon urine and minced muscle, similar determinations were carried out on blood. Known amounts of ferrocyanide solutions were added to measured volumes of whole blood and the two thoroughly mixed. For reasons at present undetermined the cyanide could not be distilled quantitatively from such mixtures. That the proteins were the interfering substances was indicated by the fact that quantitative estimations of the ferrocyanide were made readily upon protein-free blood filtrate. When, however, to the blood were added about 2 gm. of calcium chloride as a 10 per cent solution and the procedure carried through as for urine, the determination became quantitative. A heavy precipitate of calcium sulfate was formed which probably prevented the occlusion of ferrocyanide by the precipitated protein.<sup>1</sup>

It is apparent from the data of Tables II and III that with urine the procedure may be varied depending upon the amount of ferrocyanide present and upon the composition of the material with which it is mixed. When large amounts are present, a pale blue color develops in the reaction flask. This may indicate that some of the cyanide is not distilled, which would account for the lower values obtained. The amount of ferrocyanide recovered from urine is the same as that from pure solutions, but in order to make the amount recovered from minced muscle approximate that from urine or water, the time of distillation and the amount of acid must be increased. Similar changes in the procedure had much less effect when blood was used.

#### SUMMARY

A method is presented by means of which soluble ferrocyanide in amounts of 1 to 40 mg. can be determined in pure solutions and

<sup>1</sup>For this finding we are indebted to our technical assistant, Mr. M. Mittlefehldt.

in urine or minced muscle. About 98 per cent can be recovered from pure solutions, urine, or blood and about 90 per cent from muscle. This method has not been applied to the determination of insoluble ferrocyanides, hence its validity in such determinations is not known.

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**THE EFFECT OF CHANGES IN THE CONCENTRATION OF  
PLASMA ELECTROLYTES ON THE CONCENTRATION  
OF ELECTROLYTES IN THE RED BLOOD CELLS  
OF DOGS, MONKEYS, AND RABBITS**

By HERMAN YANNET, DANIEL C DARROW, AND M KATHERINE  
CARY

*(From the Department of Pediatrics, Yale University School of Medicine,  
New Haven)*

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In the present paper data pertaining to the mechanism by which erythrocytes of dogs, monkeys, and rabbits are adjusted to changes in the concentration of plasma electrolytes will be presented. The data are of particular interest as regards the permeability to cations of the membrane of red blood cells

**EXPERIMENTAL**

The technique and theoretical basis of the experimental procedures are described in detail elsewhere (1). Approximately 100 cc. per kilo of body weight of a 5 per cent solution of glucose were injected into the peritoneal cavities of the animals of one group, and a similar quantity of a 1.8 per cent solution of sodium chloride into those of another group. The amount of fluid found in the peritoneal cavities of the animals after 4 to 6 hours was approximately that injected. However, equilibration of these solutions with body fluids had resulted in a fluid with an electrolyte pattern that approximated that of interstitial fluid. In the experiments in which glucose solution was injected (hereafter designated as "glucose" experiments) equilibration required the passage of considerable extracellular electrolyte into the peritoneal cavity, resulting in a marked fall in the concentration of sodium and chloride of the serum. In the experiments in which the sodium chloride solutions were injected (hereafter designated as "saline" experiments), an increase in the concentration of sodium and chloride in the serum was produced. The change in

concentration of the various ions in the red cells 4 to 6 hours after the experimental procedure expressed the response of the erythrocyte to changes in electrolyte concentration of its surrounding medium, the blood plasma.

A total of thirty experiments was performed—fifteen on dogs, eight on monkeys, and seven on rabbits.

### *Chemical Methods*

The chemical determinations were carried out on defibrinated blood and serum. The blood was defibrinated by gentle inversion for 5 minutes in a completely filled, securely stoppered test-tube containing a small quantity of mercury. The serum was removed from blood allowed to clot under mineral oil.

Cell volume was determined on the defibrinated blood by centrifuging in capillary tubes at high speed for 20 minutes. Measurements of the height of plasma and cell volumes were made with calipers. Water content was determined by weighing before and after drying overnight in an electric oven at 105°; bicarbonate, by the method of Van Slyke and Neill (2), with 0.2 cc. of serum and 1.0 cc. of whole blood; chloride, by Patterson's method (3), with 0.5 cc. of serum and whole blood; nitrogen, by the Kjeldahl method (4), and expressed as per cent protein, the factor 6.25 being used; sodium, by the Butler and Tuthill (5) modification of the Barber and Kolthoff method, directly on serum and after removal of phosphorus from the whole blood; potassium, by Hald's modification of Shohl and Bennett's method (6). The concentrations in cells were estimated from concentrations in the serum and in whole blood by the following formula

$$C = \frac{B - (S \times (1 - V))}{V}$$

where  $C$  represented the cellular concentration;  $B$ , the concentration in whole blood;  $S$ , the concentration in serum; and  $V$ , the proportion of red cells in the blood. Since this indirect method of estimating cell concentrations tends to summate the errors inherent in the various methods, the concentrations in the cells may show a maximum error of  $\pm 6$  milli-equivalents.

## Results

The complete data, which include analyses of 70 samples of whole blood and serum, for sodium, potassium, chloride, protein, proportion of red cells by hematocrit, and, in most instances, bicarbonate and dried weight, will not be presented. The figures have been analyzed from a number of points of view but will be presented particularly to bring out the relationship between the concentrations of cations in cells and serum and the means by which this relationship is maintained.

TABLE I  
*Relationship between Concentration of Univalent Base of Serum and Cells*

	No of observations	Mean	Standard deviation	Correlation coefficient	Regression coefficient
		<i>m-eq. per kg H<sub>2</sub>O</i>	<i>m-eq. per kg H<sub>2</sub>O</i>		
Dogs....	39				
(Na) <sub>s</sub>		155.3	±10.2	+0.876	1.05 ± 0.09
(Na) <sub>c</sub>		142.6	±12.2		
Monkeys	16				
(Na + K) <sub>s</sub>		168.1	±12.8	+0.827	0.91 ± 0.16
(Na + K) <sub>c</sub>		144.6	±14.1		
Rabbits	13				
(Na + K) <sub>s</sub>		159.9	±14.0	+0.672	0.976 ± 0.22
(Na + K) <sub>c</sub>		138.7	±13.7		

(Na)<sub>s</sub> = 1.05(Na)<sub>c</sub> - 20.16 ± 5.88 for dogs; (Na + K)<sub>c</sub> = 0.91(Na + K)<sub>s</sub> - 8.46 ± 7.9 for monkeys; (Na + K)<sub>c</sub> = 0.976(Na + K)<sub>s</sub> - 19.08 ± 10.24 for rabbits. Subscripts *s* and *c* refer to concentration in serum or cells in milli-equivalents per kilo of water.

*Relation of Concentration of Univalent Cations of Serum to That of Red Cells*—The univalent cations (*i. e.* Na and K) and the anions with which they combine are responsible for about nine-tenths of the osmotic pressure of body fluids. The concept of osmotic equilibrium between body fluids, therefore, implies that, in general, variations in concentration of univalent cations of extracellular fluids are accompanied by equivalent variations in concentration of the univalent cations of intracellular fluid.

Table I gives the summary of the statistical analysis of the relation of the chief univalent cations of plasma and red cells. All

concentrations are expressed as milli-equivalents per kilo of water. When water content was not determined, it was estimated from the concentration of protein by the following formulas.

- (1) Serum water =  $0.99 - 0.8 P_s$ ,  
(2) Cell water =  $1.00 - 0.8 P_c$ .

$P_s$  and  $P_c$  represent serum and cell protein respectively, expressed as gm. per cc. (The first formula is that of Van Slyke, Wu, and McLean (7) and is confirmed by our data. The second formula is derived from our data on cell water and protein.) Since, in dogs, sodium constitutes the preponderant base of both red cells and serum, only the sodium concentrations were used. In the rabbits and monkeys, the sum of sodium and potassium was employed. Table I gives the correlation coefficient between univalent base of cells and serum together with the regression coefficients. The regression equations and their standard deviations are given beneath Table I. The statistical methods used were those recommended by Dunn (8).

The data show a close correlation between the concentrations of univalent cations in the red cells and serum of dogs and monkeys, and a significant but less close correlation in the rabbits. In all cases the magnitude of the regression coefficients (approximately 1.0) indicates that a change in serum concentration of univalent cations is associated with a practically equivalent change in cell concentration of univalent cations. The standard deviations of the regression equations ( $\pm 5.88$  in dogs,  $\pm 7.9$  in monkeys, and  $\pm 10.24$  in rabbits) indicate the distribution of the concentrations of intracellular cations at a given concentration of these cations in the serum. In other words, in somewhat more than two-thirds of the instances one could predict the concentration of preponderant cations of the cells from the concentration in the serum with an accuracy of  $\pm 6$  to 10 milli-equivalents. As was pointed out previously, owing to the summation of errors inherent in the chemical procedures employed, the concentration of cellular cations obtained by analysis may deviate from the true value by as much as 6 milli-equivalents. It is, therefore, not unlikely that the magnitude of the standard deviations of the regression equations are in great part dependent upon errors involved in the chemical procedures. With more accurate methods of analysis, a much

smaller scattering in cellular concentrations might be demonstrated.

In general, the data fit in with the accepted concepts of osmotic equilibrium in body fluids. The correlation demonstrated between the concentrations of univalent cations of serum and red cells could be brought about by shift of water, passage of cations across cellular membranes, or a combination of these two processes. The succeeding sections bring out the relative importance of these two mechanisms in preserving osmotic equilibrium between plasma and erythrocytes.

*Magnitude of Changes in Concentration of Chloride and Sodium in Serum*—The procedures in the glucose experiments decreased the concentration of serum sodium from 10 to 21 milli-equivalents per liter of serum, averaging 18 milli-equivalents. This represented a reduction of serum sodium of about 11 per cent. Reductions of chloride varied from 4 to 22 milli-equivalents per liter, averaging 18 milli-equivalents and representing an average reduction of 16 per cent. The relatively greater proportionate reduction of serum chloride than serum sodium is without doubt related to the concentration of these ions in the peritoneal fluid at the time of the second examination of the blood. The ratio of Cl:Na in the peritoneal fluid was always appreciably higher than in the serum.

In the saline experiments serum sodium increased from 7 to 25 milli-equivalents per liter of serum, averaging 19 milli-equivalents. This represented an average increase of 11 per cent. Increases of serum chloride varied from 9 to 35 milli-equivalents per liter, averaging 25 milli-equivalents and representing an average increase of 21 per cent.

*Mechanism of Maintaining Osmotic Equilibrium between Erythrocytes and Plasma*—It was shown above that the concentration of cellular cations varied directly with the concentration of plasma cations. The changes in the concentration of plasma cations were due mainly to the removal, or addition, of sodium chloride. Changes in the concentration of cellular cations were due either to a transfer of cations across the erythrocytic membrane or to a shift of water into or out of the cell. If the latter mechanism alone were responsible, the following relationships should hold.  $P_1/P_2 = E_1/E_2$  or  $E_2 = E_1P_2/P_1$ ; where  $P$  refers to the concentration of cellular

protein;  $E$  to the concentration of a given ion; subscript 1 to the initial concentration, and subscript 2 to the concentration after the experimental procedure.

In Charts I and II,  $E_2$ , as calculated from the above equation, is plotted against the concentration found by analysis. It is obvious that if there were no shift of ions across the red cell membrane, the two values should coincide and the points should fall along a straight line, having a slope of 1. (In Charts I and II, this is indicated by the solid line; the dotted lines indicate deviations

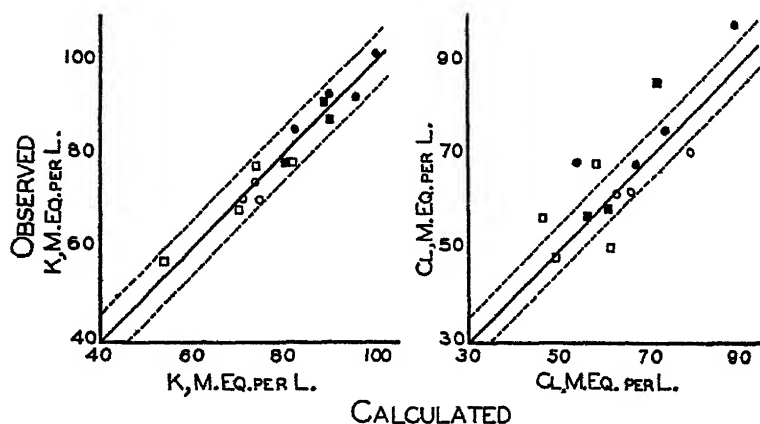


CHART I. The effect of changes in plasma electrolyte on the content of potassium and chloride in red cells of monkeys and rabbits. The values determined after the experimental procedure are plotted against the initial values after being corrected for changes in cellular water. The squares and circles represent rabbit and monkey experiments respectively. The solid symbols represent saline and the hollow symbols glucose experiments. (See the text for a detailed description.)

which may be expected owing to errors inherent in the chemical methods for determining cellular concentrations.)

Chart I shows that in rabbits and monkeys (in which potassium is the predominant cation of the red cells) the determined values for the concentration of cellular potassium coincide with the predicted values. No evidence of loss of cellular potassium occurred when plasma sodium was reduced and no increase occurred when plasma sodium was increased. The same relationship holds if sodium plus potassium, or if sodium is used. However, since

the maximum error in the chemical determination of cellular sodium is  $\pm 6$  milli-equivalents, which is almost 30 per cent of the total concentration of sodium in the red cells of these animals, small changes in sodium cannot be demonstrated. Any likely change, however, would be too small to affect the osmotic equilibrium appreciably. We may, therefore, conclude that in rabbits and monkeys, adjustment of osmotic equilibrium between red cell and plasma takes place primarily by changes in cellular water.

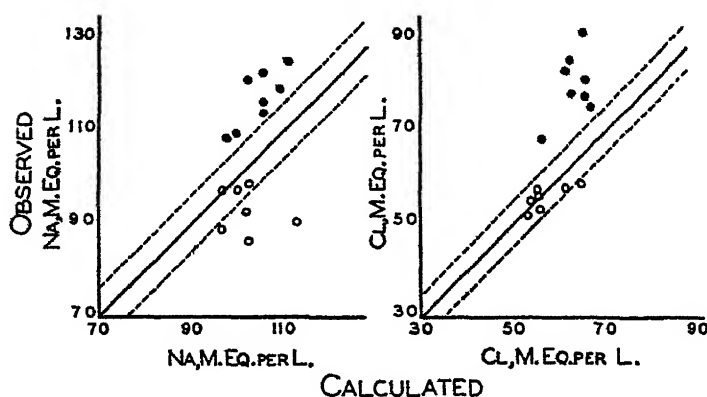


CHART II. The effect of changes in plasma electrolyte on the content of sodium and chloride in red cells of dogs. The values determined after the experimental procedure are plotted against the initial values after being corrected for changes in cellular water. The solid symbols represent saline and the hollow symbols glucose experiments. (See the text for a detailed description.)

Chart II demonstrates that in dogs (whose red cells contain sodium as the predominant cation) an increase in cellular sodium regularly occurred when plasma sodium was increased. A decrease in cellular sodium was also found in three of seven instances when plasma sodium was decreased. This observation of transfer of sodium in either direction across the erythrocytic membrane in dogs has been repeatedly confirmed in other investigations in this laboratory, including *in vitro* experiments and *in vivo* experiments during which the plasma changes in sodium were maintained for several days.

The changes occurring in cellular chloride are also charted in



like manner in Charts I and II. Little comment is necessary, since shift of chloride has been repeatedly demonstrated following various experimental procedures involving changes in serum chloride or pH. In the experiments on rabbits and monkeys alterations in serum bicarbonate were produced which were obviously related to struggling rather than changes in serum chloride and

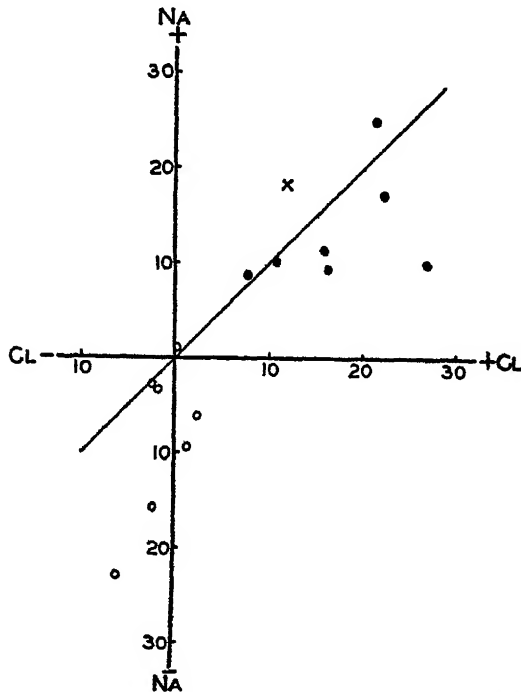


CHART III. The relationship of the changes in sodium to that of chloride in the red cells of dogs following changes in sodium and chloride in the serum. The solid and hollow circles represent the saline and glucose experiments, respectively. (See the text for a detailed description.)

sodium. For this reason the shifts of cellular chloride in rabbits and monkeys were difficult to interpret and were thought to be related to changes in pH rather than fluctuations in the concentration of serum chloride or sodium.

Since in the dogs struggling was minimal and changes in serum bicarbonate were slight, the changes in cellular chloride are related

to the changes in serum chloride and sodium. In Chart III the changes in cellular sodium are plotted against the changes in cellular chloride. It will be seen that in the glucose experiments decreases in cellular sodium were accompanied by minimal changes in cellular chloride. In the saline experiments the increases of cellular sodium were associated in every instance but one by greater increase in cellular chloride. Since the injection of a solution of sodium chloride increased the concentration of serum chloride more than serum sodium, the greater increase in cellular chloride than cellular sodium might be expected. In the one experiment (marked X) in which cellular sodium increased more than cellular chloride, a solution which contained 80 milli-equivalents of sodium bicarbonate and 220 of sodium chloride was injected. In this instance, serum chloride and sodium increased about equivalent amounts

#### DISCUSSION

Studies of the changes in whole blood saturated at various tensions of carbon dioxide reveal that osmotic and ionic equilibrium between erythrocyte and plasma are adjusted by shifts of anions (chloride and bicarbonate) and water (9). Analogous changes have been described by Harkins and Hastings (10) in response to the injection of hydrochloric acid in dogs. Wakeman, Eisenman, and Peters (11), furthermore, have demonstrated a similar process of equilibrium, when monovalent salts, like sodium and potassium chloride, are added to human blood. On the other hand, Hamburger and Bubanovic (12) and Hamburger (13) showed that the red cells of beef blood respond to changes in sodium and potassium concentration of the serum, not only by shifts of water but also by the passage of these cations across the erythrocytic membrane. A similar shift of potassium has been demonstrated by Kerr (14) in dog, sheep, and beef red cells. The same author failed to duplicate these findings in human erythrocytes except when watery solutions were substituted for serum. The present data demonstrate that, in dogs, changes in plasma sodium may be accompanied by changes in erythrocytic sodium. In monkeys and rabbits, however, similar plasma changes, under identical experimental conditions, failed to produce a similar shift of sodium or potassium across erythrocytic membranes. At first glance, these varying results seem con-

tradictory Some of these apparent discrepancies may be dependent on differences in experimental techniques and analytical methods. Probably of greater importance, however, are the differences in electrolytic configuration of the red cells of the animals studied.

In Table II average sodium and potassium concentrations of the red cells of dog, sheep, cow, rabbit, monkey, and man are presented. The red cells of these animals fall into two groups. In the first (dog, sheep, and cow) the sodium and potassium concentrations expressed in terms of water content tend to approximate the concentration of these ions in serum. In the second group (monkey, rabbit, and man) the preponderant cation is potassium, while sodium is present in relatively small amounts. It will be

TABLE II  
*Concentration of Sodium and Potassium (Milli-Equivalents per Kilo of Water) in Erythrocytes As Related to Experimental Evidence of Cation Permeability*

	Sodium	Potassium	Cation permeability
Dog ..	143	8	+
Sheep (Kerr (14)). . . . .	110	46	+
Cow (Kerr) . . . . .	116	23	+
Rabbit... . . . .	31	108	—
Monkey... . . . .	32	112	—
Human (Peters (15)) . . . . .	25	114	—

noted that experimental evidence of shift of sodium or potassium across the erythrocytic membrane, as far as the available literature and the present data are concerned, has been demonstrated only for the former group, *i e* dog, sheep, and cow. Apparently, erythrocytes with an electrolytic pattern resembling plasma exhibit greater permeability of the cellular membrane to cations than those with an electrolyte pattern differing widely from that of plasma. These findings, however, should probably not be interpreted as indicating that cells containing sodium as the preponderant base are freely permeable to cations nor that cells containing potassium as the preponderant base are absolutely impermeable to cations.

Since analyses of tissues, except the blood, of the animals men-

tioned above indicate that practically all cells contain potassium as the preponderant base, the common assumption that cellular membranes are, in general, relatively impermeable to cations is in agreement with the results obtained on erythrocytes of comparable electrolyte pattern.

The anions of the red cells are chiefly chloride, bicarbonate, phosphate (chiefly organic), and protein. Approximately two-thirds of the cell base is bound by chloride and bicarbonate, the remainder being about equally divided between phosphate and protein. The data available in this study limit the discussion to chloride and bicarbonate. The reciprocal relationships of these anions in the maintenance of acid-base equilibrium in the red cell were demonstrable in the results of the saline experiments on dogs. The increase in cell sodium was in every experiment associated with a somewhat greater increase in cell chloride. In every case but one this discrepancy was partially balanced by a decrease in cell bicarbonate. In one experiment the dog was injected with a solution which differed from the 1.8 per cent sodium chloride solution used in the other experiments, in that 80 milli-equivalents of chloride were replaced by an equivalent amount of bicarbonate. In this animal the increase in cellular sodium was greater than that of cellular chloride, while the cellular bicarbonate in this instance increased so that balance was maintained. In spite of the technical limitations obtaining in a study of this kind, the tendency for the increase in cell sodium to be almost balanced by an equivalent increase in the sum of chloride and bicarbonate of the cell is a striking feature of most of these experiments.

#### SUMMARY

Studies of the changes in the red cells resulting from alterations in electrolyte concentration in plasma of dogs, monkeys, and rabbits are reported.

A statistical analysis of the results showed a high degree of correlation between the univalent cations of the red cells and plasma. The magnitudes of the regression coefficients indicated that a given change in concentration of univalent cations in serum was accompanied by an equivalent change in the univalent cations of the red cells. These results fit the generally accepted concepts of osmotic equilibrium in body fluids.

Shift of water across the red cell membrane constituted the chief, if not the only, demonstrable mechanism of adjusting osmotic equilibrium in the monkeys and rabbits. In the dogs, however, besides the shift of water, passage of sodium across the cell membrane could also be demonstrated.

A review of the available literature on the permeability of red cells of different animals, together with the data presented, leads to the conclusion that erythrocytes containing sodium as the preponderant base are permeable to sodium. A similar permeability of erythrocytes containing potassium as the preponderant base has not been demonstrated.

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## A MONOHYDROXPALMITIC ACID IN BUTTER FAT

By A. W. BOSWORTH AND G. E. HELZ

*(From the Laboratory of Physiological Chemistry, the Ohio State University, Columbus)*

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During an investigation concerning the unsaturated fatty acids present in butter fat, certain preparations of unsaturated fatty acids were isolated, which seemed to contain a saturated fatty acid with properties quite different from those of the ordinary saturated acids. Upon investigation it was found that this unknown acid was an optically active hydroxy acid which was liquid at ordinary room temperature. This paper is a report of these observations

The material used comprised the methyl esters of butter fat as prepared by Bosworth and Sisson (1), except that they were redistilled at a pressure of 15 mm. As the purpose of our investigation was to isolate and identify the optically active acid present in our fractions, the first step was to ascertain the fractions in which this acid was present. This was done by observing the angular rotation of polarized light caused by the different fractions. The data thus secured will be found in Table I.

From 500 to 750 gm. of the esters from each of Fractions 24 to 29, inclusive, were saponified and then subjected to the barium soap-benzene separation. This was done in order to remove the solid saturated acids and most of the unsaturated acid containing one double bond. The barium soaps which were soluble in benzene were converted to acids and these acids were then subjected to the lithium soap-acetone separation (2) in order to remove the arachidonic acid. The insoluble lithium soaps thus obtained were converted to acids and then subjected to the lead soap-ether separation to remove as far as possible the ordinary saturated fatty acids remaining after the original barium soap-benzene separation. The ether-soluble lead soaps were converted to acids and subjected

## 490 Monohydroxypalmitic Acid in Butter Fat

to the barium soap-benzene separation for further purification. A small amount of insoluble barium soaps was precipitated. The benzene-soluble barium soaps were converted to acids and these acids, representing a total of 149 gm., had mean molecular weights ranging from 243 to 267 and iodine numbers ranging from 65.4 to 82.5.

TABLE I

*Optical Activity of Original Fractions of Methyl Esters of Fatty Acids from Butter Fat*

A 400 mm tube was used.

Fraction No	Boiling range at 15 mm	Rotation	Fraction No	Boiling range at 15 mm	Rotation
	°C	degrees		°C	degrees
22	155-160	0 00	27	180-185	+0 49
23	160-165	+	28	185-190	+0 35
24	165-170	+0 21	29	190-195	+0 28
25	170-175	+0 35	30	195-200	Cloudy
26	175-180	+0 55	31	200-205	0 00

TABLE II

*Results Obtained from Examination of Hydroxy Acid Separated from Several Fractions of the Methyl Esters of the Fatty Acids from Butter Fat*

Fraction No	Weight of acids	Mean mol wt	M p	I No Hanus, 30 min	Acetyl value	Specific rotation*
	gm		°C.			degrees
24	8	268	16 5		151	+2 43
25	14	269	16 5		167	
26	26	270	17 0	0 62	173	+2 47
27	22	271	17 0	0 48	175	+2 45
28	18	273	17 0		165	+2 47
29	9	273	17 5		152	+2 40

\* A known weight (about 5 gm ) of the acid was dissolved in chloroform to make 50 cc The rotation was observed by the use of a 400 mm tube

It is quite evident therefore that the preparations are mixtures of saturated and unsaturated acids. These acids were next hydrogenated, platinum being used as a catalyst. The reduced acids were then subjected to a lead soap-ether separation to remove the saturated acids formed by hydrogenation. The ether-soluble

lead soaps were converted to acids and examined. The results obtained are given in Table II.

The figures for the molecular weights and acetyl values indicate that the acid we have separated is a monohydroxypalmitic acid of fairly high degree of purity (theory, mol. wt. 272, acetyl value 178). The melting points would seem to indicate that the acid we have separated differs from any of the monohydroxypalmitic acids previously reported. The fact that these preparations have a slight iodine number, indicating the presence of traces of unsaturated acids, throws some doubt as to the true melting point of the acid we have identified.

The acids as obtained from the several fractions were united. Upon analysis the following figures were obtained.

	Found	Theory for $C_{16}H_{32}O_2$
	<i>per cent</i>	<i>per cent</i>
Carbon . . . . .	71 03	70 52
Hydrogen . . . . .	12 06	11 85
Oxygen by difference. . . . .	16 91	17 63

As our preparations were not purified by a process of crystallization, the percentages of the elements as determined are not as close to the theoretical as might be desired. They do show, however, that we are dealing with a monohydroxy acid.

All of the acid remaining was subjected to reduction by hydriodic acid according to the method as outlined by Crowder and Anderson (3). The reduced acid, after crystallization from acetone, had a melting point of 62.5° (palmitic acid, 62.6–63°). When some of the substance was mixed with some pure palmitic acid, there was no change in its melting point. The molecular weight as determined by titration with 0.1 N alkali was found to be 256 (palmitic acid, 256.3). The reduced acid was identified as palmitic acid and the original acid from which the palmitic acid was derived was identified as a monohydroxypalmitic acid.

#### SUMMARY

An optically active monohydroxypalmitic acid has been separated from butter fat. The lead soap of this acid is soluble in ether and the barium soap is soluble in benzene.



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The authors wish to acknowledge their indebtedness to the M and R Dietetic Laboratories, Inc., for financial assistance. They also express their appreciation to Dr. C. S. Smith, Chairman of this department for his encouragement.

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## THE SPECIFIC ROTATION OF *L*-CYSTINE IN RELATION TO DEGREE OF NEUTRALIZATION AND pH

By GERRIT TOENNIES, THEODORE F. LAVINE, AND MARY A  
BENNETT

*(From the Lankenau Hospital Research Institute, Philadelphia)*

(Received for publication, September 23, 1935)

The importance of polarimetric standardization of cystine used in biochemical work has first been emphasized by Marston and Robertson (1). Standard values in *N* HCl have been established (2). However, data on the specific rotation of cystine at different acidities have recently been published from two sources (3, 4), which in both cases evidently were obtained on material containing about 15 per cent of optically inactive cystine. Since these figures might inadvertently be used as reference data, especially in connection with oxidation of optically active cysteine, we submit the corresponding data obtained by us on *L*-cystine of 98 to 100 per cent purity. The determinations covering the whole pH range were made in 1930, when it was found that the lithium salt of *L*-cystine (5) was a suitable starting material, especially for the production of a supersaturated solution in the isoelectric range. The data obtained—not published previously, since the investigation aiming at establishing individual specific rotations for the different cystine ions remained uncompleted—are now presented, together with recent determinations obtained on pure cystine and by reoxidation of cysteine according to Pirie's method (4).

### EXPERIMENTAL

The particular preparation of lithium cystinate used is one described in an earlier paper ((5) p. 211) where it is shown to consist to 98 to 99 per cent of the levorotatory modification. Optical rotation, temperature, and colorimetric pH were determined on 25 cc. solutions of the lithium salt, 0.005 *M* in cystine and containing definite amounts of HCl or LiOH. Approximate pH values

were obtained by visual comparisons by use of Clark's (6) sulfonphthalein indicators and, for the alkaline range beyond pH 10, LaMotte's nitro yellow, sulfo orange, and violet (7), with Clark and Lubs and Sørensen buffer mixtures (6) as standards. Table I gives the relations between degree of neutralization and pH, while specific rotation and pH are plotted in Fig. 1. The isoelectric zone indicated by the range of constant specific rotation is seen to be in good agreement with the isoelectric zone as indicated by the range of constant minimum solubility (9, 10). Included for comparison are the data of Pirie (4). His values are for 20° and a cys-

TABLE I  
*Relation between Degree of Neutralization and pH of Cystine Solutions.  
Cystine 0.005 M*

LiOH	HCl	pH	LiOH	HCl	pH
M	M		M	M	
0 010	0 100	1 1	0 010	0 009	7 2
0 010	0 080	1 3	0 010	0 008	7 6
0 010	0 060	1 5	0 010	0 006	8 3
0 010	0 040	1 7	0 010	0 005	8 45
0 010	0 030	1 8	0 010	0 004	8 7
0 010	0 022	2 0	0 010	0 003	9 0
0 010	0 020	2 1	0 010	0 002	9 15
0 010	0 018	2 2	0 010	0 001	9 25
0 010	0 016	2 4	0 010	0 000	9 9
0 010	0 014	2 55	0 012	0 000	10 8
0 010	0 012	2 85	0 014	0 000	11 4
0 010	0 0106	3 6	0 016	0 000	11 5
0 010	0 010	5 7	0 018	0 000	11 6

tine concentration of 0.0083 M, while the concentration in his cysteine oxidations is 0 0064 M. Oxidation of free *l*-cysteine (11), in an equivalent amount of HCl, with H<sub>2</sub>O<sub>2</sub> and Cu under the conditions of Pirie ((4) Fig 1, Experiment 1) gave within less than 15 minutes  $[\alpha]_{\text{H}_g}^{26} = -322^\circ$  at pH 2.2 to 2.3. With a temperature coefficient of  $d[\alpha]/dt = +1.5^\circ$ , obtained on 0 0083 M cystine in HCl neutralized to pH 2.0 ( $[\alpha]_{\text{H}_g} = -312.1^\circ$ ,  $-301.3^\circ$ , and  $-293.1^\circ$  at 19.3°, 26.7°, and 32.05°), this value corresponds to  $-316^\circ$  at 30°, as compared with  $-311^\circ$  read from Fig. 1, in good agreement with the estimated purity of the material used in obtaining the

data of Fig. 1. On the other hand, on the basis of the same temperature coefficient the values of Pirie at pH 2 to 2.5 indicate a stereochemical purity of about 85 per cent. Of a similar order of

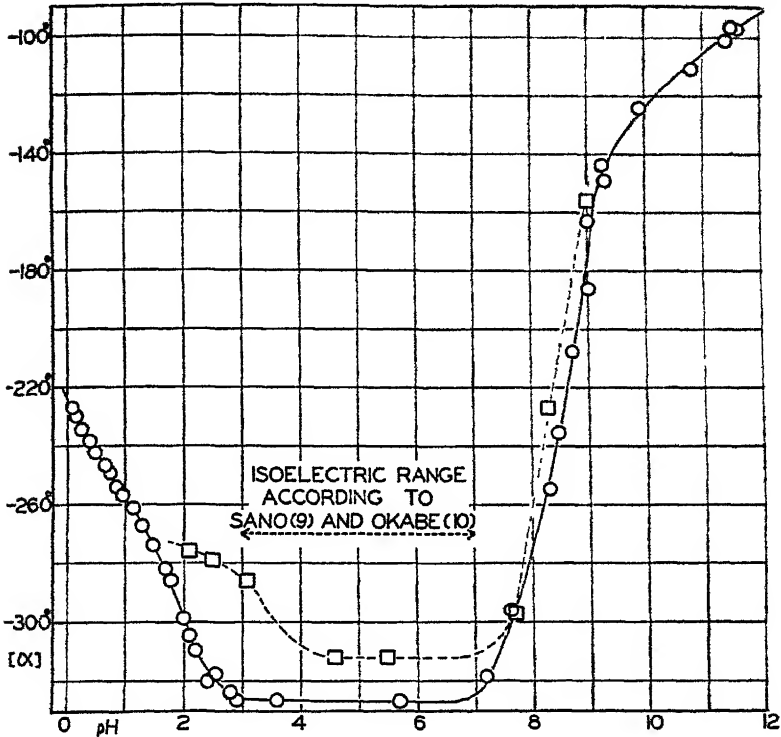


FIG. 1. Specific rotation of *l*-cystine and pH. Cystine, 0.005 M. Average temperature,  $30.0^\circ \pm 0.5^\circ$  for pH 1.1 to 11.6 and  $31.0^\circ \pm 0.5^\circ$  for pH 0 to 1. pH values between 0 and 1 are calculated from the HCl concentration (0.14 to 1.02 M) according to the activity coefficients of HCl (8), the cystine concentrations being disregarded, while above 1 the pH was estimated colorimetrically. For the determinations below pH 1 free cystine was used instead of the Li salt. The broken line curve refers to the data of Pirie (4) for cystine 0.0083 M, temperature  $20^\circ$ .

purity is the optically active cystine used by Lutz and Jirgensons (3). In agreement with the finding of Andrews (12) that there is no significant difference in the specific rotation of cystine between

concentrations of 0.4 and 1.0 gm. per 100 cc. of N HCl we find (2) for a 1 per cent concentration  $[\alpha]_{\text{H}_g}^{31.5} = -200^\circ$ , while a determination in 0.4 per cent concentration gave  $[\alpha]_{\text{H}_g}^{31.5} = -198.5^\circ$ . The determinations of Lutz and Jirgensons for the latter concentration yield  $[\alpha]_{\text{H}_g}^{31.5} = -169^\circ$ ; *i.e.* 15 per cent lower than our own value.

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## DETERMINATION OF THE STEREOCHEMICAL PURITY OF *L*-CYSTEINE

BY GERRIT TOENNIES AND MARY A. BENNETT

(*From the Lankenau Hospital Research Institute, Philadelphia*)

(Received for publication, September 23, 1935)

The interest of all studies on cysteine, cystine, and kindred compounds is ultimately related to the problem of their biological significance. In view of the general biological specificity of stereoisomers—which with regard to the metabolism of cystine was experimentally demonstrated by the work of du Vigneaud and his associates (1)—reliable criteria for the polarimetric purity of these compounds are essential. Andrews (2) and Toennies and Lavine (3) have established standard conditions and values<sup>1</sup> for the polarimetric examination of *L*-cystine. The difficulties in the way of obtaining a constant of similar accuracy for the naturally occurring cysteine<sup>2</sup> are due to the fact that the numerical value of its specific rotation is about 20 times smaller than that of cystine, so that high concentrations have to be used in order to get a satisfactory precision. Even then, however, the presence of, for instance, less than 0.5 per cent of *L*-cystine—easily formed by oxidation—would cause a decrease of 10 per cent in the absolute rotation value of dextrorotatory cysteine, thus simulating a content of 10 per cent of optically inactive cysteine. It seems more rational to establish iodometrically (8) or colorimetrically (9) the —SH content of the sample to be tested and to determine the specific rotation of the cystine formed by quantitative oxidation. Pirie (6) demonstrated polarimetrically that cysteine is rapidly and quantitatively oxidized to cystine at pH 2 by hydrogen peroxide in

<sup>1</sup> Recently confirmed by Brown and Lewis (4).

<sup>2</sup> Belonging to the natural series of amino acids, it should be called *L*-cysteine. Its rotation shifts in a negative direction with decreasing acidity (5, 6) in agreement with the rule of Lutz and Jirgensons (7) for the natural series

the presence of copper ion. In that pH range, however, the solubility of cystine is low and, besides, the specific rotation changes greatly with small variations in pH (10). We found that by increasing the copper concentration the peroxide oxidation can be carried out conveniently in *N* HCl and 1 per cent concentration (the standard conditions for polarimetry of cystine (2, 3)), and that pure optically active cysteine produces a value coinciding with that of pure *l*-cystine.

#### EXPERIMENTAL

*Effect of  $\text{Cu}^{++}$  on Speed of Oxidation in *N* HCl*—Commercial cysteine hydrochloride (94 per cent according to acid content, and 6 per cent  $\text{H}_2\text{O}$ ) was oxidized in *N* HCl with  $\text{H}_2\text{O}_2$  in the presence of varying amounts of  $\text{CuSO}_4$ . The reaction was followed by polarimetric readings of  $\alpha_{\text{H}_2\text{O}}$ . The results are given in Table I.

After passing through the maximum level, the rotation declines again slowly, down to a stable level: in Experiment I, 78.4, 73.9, 63.4, 43.6, 42.2, 37.1 per cent after 21, 28, 45, 95, 101, and 125 hours respectively, in Experiment II, 57.4, 57.8, 57.6, 57.6 per cent after 20, 26, 44, and 83 hours, and in Experiment III, 52.3, 52.1, 51.7, 51.3 per cent after 18, 24, 42, and 90 hours. The reaction velocity appears, just as in Pirie's (6) experiments at pH 2, proportional to the  $\text{Cu}^{++}$  concentration.

*Free Cysteine*—In further experiments the free amino acid instead of the hydrochloride was used. It is easily obtained as follows:

Dissolve 10 gm. of cysteine hydrochloride in 7 cc. of water by gentle warming. If any insoluble material is present, dilute with 30 cc. of ordinary alcohol and filter. Dilute the filtrate with 130 cc. of alcohol and add immediately, with shaking, 5.3 cc. of pyridine. Precipitation of free cysteine occurs immediately in crystalline form. Leave for 2 hours near  $0^\circ$  and filter on a Buchner funnel. Wash carefully with 25 cc. portions of chloroform at least six times. The precipitate should then be free of pyridine odor and the  $\text{Cl}^-$  reaction should be negative. The low solubility of a silver salt of cysteine, even in acid solution, is apt to simulate a  $\text{Cl}^-$  reaction in the cold. Dissolve a small sample of the cysteine in dilute nitric acid, heat to boiling, and add silver nitrate. Unless the solution is too concentrated, the silver salt of cysteine will not

crystallize until the solution cools. After 1 night under a high vacuum in the presence of  $P_2O_5$  the substance is dry.<sup>3</sup> There is no evidence of decomposition or oxidation if the substance is kept in a tightly closed container near  $0^\circ$  in the dark. The yield is 80 to 85 per cent of the theory.

*Effect of Variation of  $H_2O_2$  Concentration on Maximum Amount of Cystine Formed*—Free cysteine prepared, in the manner described, from Merck's cysteine hydrochloride which is manufactured from pure *l*-cystine,<sup>4</sup> was used in this group of experiments. The iodine consumption found by the method of Lavine (8) was

TABLE I  
*Oxidation of Cystine in  $\pi$  HCl by  $H_2O_2$  in Presence of Varying Amounts of  $CuSO_4$*

The experiments were carried out at  $28-29^\circ$ . Cystine  $0.0833$  M corresponding to  $1.00$  gm. of cystine per  $100$  cc;  $H_2O_2$   $0.192$  M (Experiment I),  $0.176$  M (Experiment II and III) (calculated for the oxidation of cystine to cystine  $0.0417$  M);  $[\alpha]$  expressed in terms of per cent of theoretical value of *l*-cystine (3) corresponding to total cysteine present

Experiment No	Cu <sup>++</sup>	Oxidation time							
		5 min	10 min	15 min	20 min	25 min	70 min	140 min	210 min
		[ $\alpha$ ]							
	mole per liter	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
I	$1 \times 10^{-4}$	7.2	17.1	24.7	31.3	45.9	62.8	76.5	83.2
II	$1 \times 10^{-3}$	82.7	88.5	91.0	91.6	92.2	93.2	93.2	
III	$5 \times 10^{-3}$	93.4		93.4					

$100.7 \pm 0.1$  per cent of the theoretical amount for oxidation to cystine. The composition of the solutions—made up in  $25$  cc. volumetric flasks and read polarimetrically ( $\alpha_{H_2}$ ) in a  $2$  dm. tube—is indicated in Fig. 1. The individual points on the curves were obtained by combining groups of single polarimeter and thermometer readings, extending over  $10$  minute periods, into average values, and expressing the results in terms of per cent of the corresponding polarimetric values calculated (3) for complete conversion of the cysteine present into cystine. A consistent increase of

<sup>3</sup> Adhering traces of pyridine, revealed by its odor after  $CHCl_3$ , has disappeared, are best removed over  $H_2SO_4$  *in vacuo*.

<sup>4</sup> Personal communication from Merck and Company, Inc.



the peak value with decreasing excess of hydrogen peroxide, and approaching 100 per cent, is evident. The relation is illustrated in the inset, where the maximum value of each curve is plotted against the corresponding peroxide excess (the semilogarithmic scale is chosen merely as an empirical means of demonstration, as it would be futile with the present data to attempt evaluating the actual function involving presumably several simultaneous and consecutive reactions).

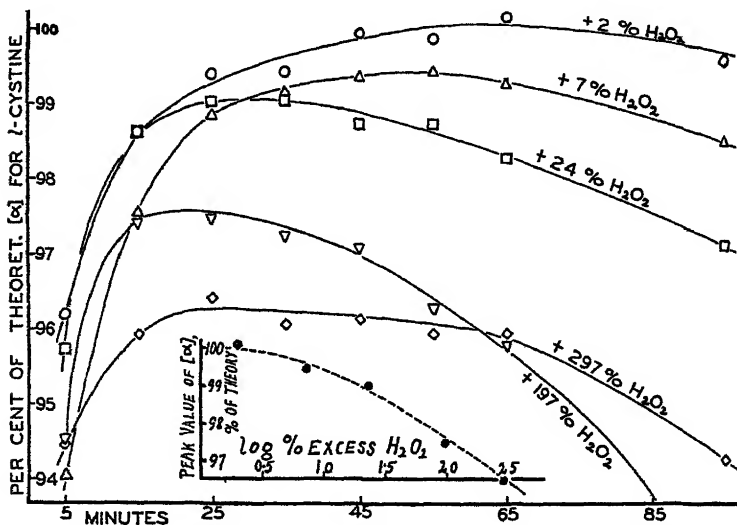


FIG. 1 Oxidation of cysteine with varying amounts of hydrogen peroxide. HCl 1 M; cysteine 0.084 M, corresponding to 1 gm of cysteine per 100 cc.;  $\text{CuSO}_4$  0.003 M; temperature  $26.8^\circ \pm 1^\circ$ ;  $\text{H}_2\text{O}_2$  0.042  $(1 + (n/100))\text{M}$ , where  $n$  = per cent given on the individual curves. The inset shows the maximum rotation plotted against  $\log n$ .

These experiments indicate that the stereochemical purity of *l*-cysteine may be measured with an accuracy of about  $\pm 0.25$  per cent by first establishing the  $-\text{SH}$  content (8, 9) and then determining in a 2 dm tube the maximum optical rotation reached in the course of 1 hour by a solution of 0.25 gm. of cysteine in 25 cc. of  $\text{N HCl}$  and 0.003 M  $\text{Cu}^{++}$  in the presence of hydrogen peroxide, about 2 per cent in excess over the amount calculated for complete conversion into cysteine.

*Optical Rotation of l-Cysteine Itself*—The substance characterized by the preceding experiments gave  $[\alpha]_{\text{H}_2\text{O}}^{26} = +9.69^\circ \pm 0.05^\circ$ ; another preparation gave  $+9.69^\circ \pm 0.09^\circ$ ; and the corresponding values for sodium light averaged  $[\alpha]_{\text{D}}^{26} = +7.6^\circ \pm 0.1^\circ$ . The solutions were 1 M cysteine (12.1 per cent) and 1 M HCl, density at  $26^\circ = 1.053$ . A temperature coefficient of approximately  $d\alpha'/dt = -1$  per cent was found between  $23$ – $33^\circ$ .

The  $[\alpha]_{\text{D}}$  value is distinctly higher than the one recorded by Bergmann and Michals (5) which was obtained under similar conditions. Exact measurements in lower concentration comparable with the data of other authors (11) were not made, since the maximum precision attainable in a 1 per cent concentration in a 2 dm. tube would be only about  $\pm 5$  per cent, and since rigid exclusion of oxygen would be necessary to obtain a significant value of even that limited accuracy. In the closed tube a slow oxidation takes place over several hours, the extent of which is limited evidently by the amount of dissolved oxygen: in a 12 per cent solution  $\alpha$  decreased by about 3 per cent in about 5 hours, while in a 1 per cent solution the decrease was about 17 per cent in 5 hours. Two independent experiments on two preparations gave by extrapolation to zero time  $[\alpha]_{\text{H}_2\text{O}}^{25} = +10.6^\circ \pm 0.1^\circ$  in solutions of 1 gm. of cysteine per 100 cc. of 1 N HCl.

Thanks are expressed to Merck and Company, Inc., for donations of cysteine hydrochloride.

#### SUMMARY

1. The oxidation of cysteine by hydrogen peroxide in N HCl can be catalyzed to conveniently measurable rates by copper ion concentrations of the order of  $10^{-3}$  M

2. On this basis conditions were established for the complete conversion of cysteine into cystine for the purpose of determining the stereochemical purity of cysteine preparations with an accuracy of approximately  $\pm 0.25$  per cent.

3. Data are given for the preparation of free *l*-cysteine from the hydrochloride and for its optical rotation.

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# THE PRODUCTION OF A DEFICIENCY INVOLVING CYSTINE AND METHIONINE BY THE ADMINIS- TRATION OF CHOLIC ACID\*

By ABRAHAM WHITE

(From the Department of Physiological Chemistry, Yale University,  
New Haven)

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The assumption that the taurine of the bile salts has its origin in dietary cystine is founded, in general, on the *in vitro* synthesis of taurine from cystine by Friedmann in 1903 (1), and on the investigations with animals which have, in the majority of instances, involved determinations of the influence of exogenous cystine on the concentration of bile salts<sup>1</sup> The evidence for the synthesis is based upon reactions which subsequent investigators have been unable to repeat (4)<sup>2</sup> The data from *in vivo* studies ob-

\* This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association.

A part of the data of this study was reported at the Leningrad-Moscow meeting of the Fifteenth International Physiological Congress under tenure of an International Physiological Congress Fund Fellowship

<sup>1</sup> For a discussion of this evidence, reference should be made to the reviews of Lewis (2, 3).

<sup>2</sup> Friedmann's transformation of cystine into taurine involved the oxidation of cystine to cysteic acid and a subsequent decarboxylation of the latter compound It is the decarboxylation reaction which does not, apparently, proceed as described by Friedmann. Lewis and Lewis (5), in a foot-note, have mentioned the only successful decarboxylation the author has been able to find to support Friedmann's work Gortner and Hoffman (4) suggested that inasmuch as Friedmann's cysteic acid was prepared through the copper salt, while theirs was not, "It may be that copper catalyzes the reaction" Since Gortner and Hoffman did not test this possibility, Dr. H. B. Vickery and the author have attempted to repeat Friedmann's synthesis exactly as described by the latter investigator However, attempts to decarboxylate cysteic acid, prepared either through the copper salt or by direct crystallization, were unsuccessful. This decarboxylation reaction is receiving further attention.

tained by determination of the organic sulfur of the bile or by colorimetric estimation of bile salts can be criticized on the grounds of specificity of reactions as well as interpretation of the results.

In order to secure additional information on the origin of taurine and the relation of the latter compound to dietary cystine, a new method of approach has been employed. A nutritional deficiency in cystine has been produced by the addition of cholic acid to a diet somewhat low in this sulfur-containing amino acid. As a working hypothesis, it was assumed that a portion of the cholic acid ingested would be conjugated with taurine and, therefore, indirectly impose on the growing animal an added metabolic demand for cystine.

#### EXPERIMENTAL

Male rats at weaning were caged singly and given a basal diet consisting of technical casein<sup>3</sup> 6, inorganic salts<sup>4</sup> 4, sucrose 15, starch 50, and lard 25 per cent. Throughout the investigation, each animal received a daily supplement of 400 mg of yeast<sup>5</sup> and 100 mg. of cod liver oil. The amount of the sulfur-containing amino acids in the diet was limited in order to accentuate the effects of the cholic acid. Nevertheless, the quantity and nutritional quality of the protein supplied by the casein and the yeast supplement were adequate for the promotion of good growth for relatively long periods of time. This is shown in Chart I which, together with Chart II, presents in graphic form results representative of those obtained in the present investigation.

When the animals had reached a body weight of 70 to 85 gm. each, the ration was changed to one prepared by incorporating 816 mg of cholic acid<sup>6</sup> in 100 gm of the basal diet. Animals transferred to this diet ceased growing and gradually lost weight,

<sup>3</sup> Technical casein (Lister Brothers, New York).

<sup>4</sup> Osborne and Mendel salt mixture (Osborne, T B, and Mendel, L B, *J. Biol. Chem.*, **37**, 572 (1919))

<sup>5</sup> Product of the Northwestern Yeast Company.

<sup>6</sup> Two specimens of cholic acid were used in this investigation; one was prepared from ox bile by the method of Schryver (6), and the other was a product of Hoffmann-La Roche, Inc. The purity of each sample was established by melting point and by mixed melting point with a recrystallized, analytically pure (carbon and hydrogen analysis) sample of cholic acid. Both preparations of cholic acid behaved the same in these studies

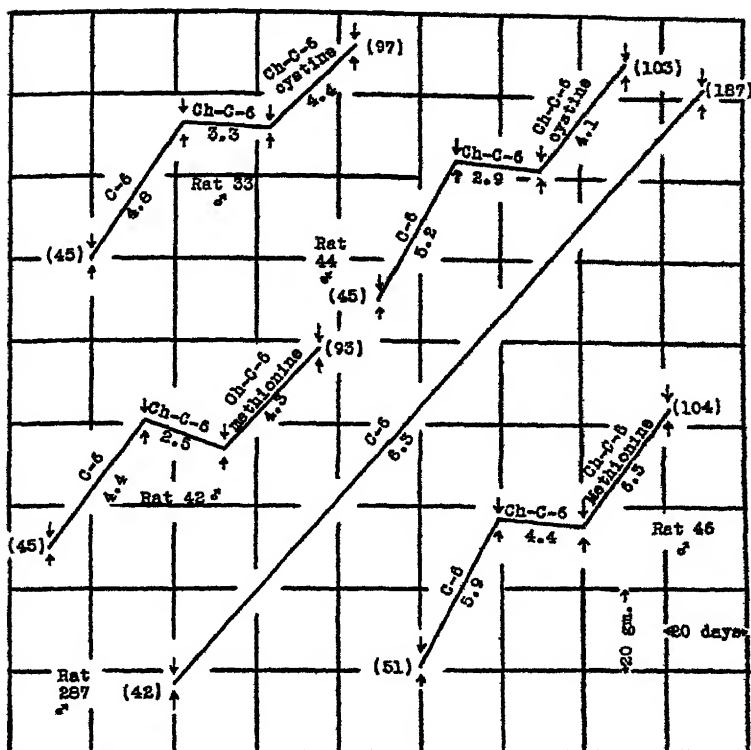


CHART I. Growth on basal diet, on basal diet with added cholic acid, and on cholic acid-containing basal diet with the addition of cystine or methionine. The diet employed in any portion of an experiment is indicated by the two downward arrows representing the beginning and end of a period. Diet C-6, basal diet; Diet Ch-C-6, 816 mg of cholic acid per 100 gm. of basal diet. The quantities of the supplements fed are presented in the text. The average daily food consumption in gm for the corresponding interval is shown by the figures between the upward arrows. The initial and final body weights are presented in parentheses. The cystine, methionine, and basal diet curves are representative of groups of fourteen, four, and five animals, respectively.

the average daily decline in a group of twenty rats being 0.3 gm. Various substances were then tested for their abilities measurably to relieve the deficiency imposed by the cholic acid. Either

*l*-cystine<sup>7</sup> or *dl*-methionine, in quantities of 360 mg. (called 3 equivalents<sup>8</sup>) and 450 mg. (3 equivalents) respectively, added to 100 gm. of the basal diet already containing the cholic acid, effected immediate and definite resumption of growth. In a group of fourteen animals receiving the cystine supplement, the average daily weight increment was 1.0 gm., and with four animals given methionine supplements, the average daily gain in weight was also 1.0 gm. On the other hand, sulfur added in the form of either taurine (4 equivalents) or sodium sulfate (5 equivalents) to 100 gm. of the cholic acid-containing diet was incapable of alleviating the deficiency caused by the cholic acid, notwithstanding the fact that these materials supplied more sulfur than did the effective quantities of cystine and methionine. The amino acid glycine (3 equivalents) was also without effect under these experimental conditions. The latter compound was studied, inasmuch as the relative importance of taurocholic and glycocholic acids in rat bile has not been established. Furthermore, an amino acid digest prepared from casein (7) was also tested in order to determine whether a small increase in the intake of amino acid nitrogen in general would stimulate growth. Assuming an average molecular weight of 150 for the amino acids of the casein hydrolysate, 3 equivalents (450 mg.) of the digest were added to 100 gm. of the basal diet containing the cholic acid. This supplement was likewise incapable of stimulating growth. Finally, a group of animals was permitted to ingest the cholic acid-containing diet for a period of time, following which the compound was removed from the ration. The prolonged ingestion of cholic acid does not produce any evident impairment in the subsequent capacity of the animal to grow because, following the removal of this substance from the

<sup>7</sup> The *l*-cystine was prepared from hair. The *dl*-methionine was the synthetic compound obtained from Organic Chemical Manufactures Division, University of Illinois. The taurine and the glycine were products of the Eastman Kodak Company. The purity of the glycine was established by Kjeldahl nitrogen determination; the other three compounds yielded the theoretical quantities of sulfur on analysis by the Parr bomb procedure. The sodium sulfate employed was Baker's anhydrous sodium sulfate, c. p. quality.

<sup>8</sup> 120 mg of cystine are termed 1 equivalent. For the other supplements, the molecular weight of each compound in mg is called 1 equivalent; thus 450 mg of methionine = 3 equivalents, 710 mg. of  $\text{Na}_2\text{SO}_4$  = 5 equivalents, etc.

diet, growth is resumed at approximately the normal rate observed for animals ingesting the basal diet.

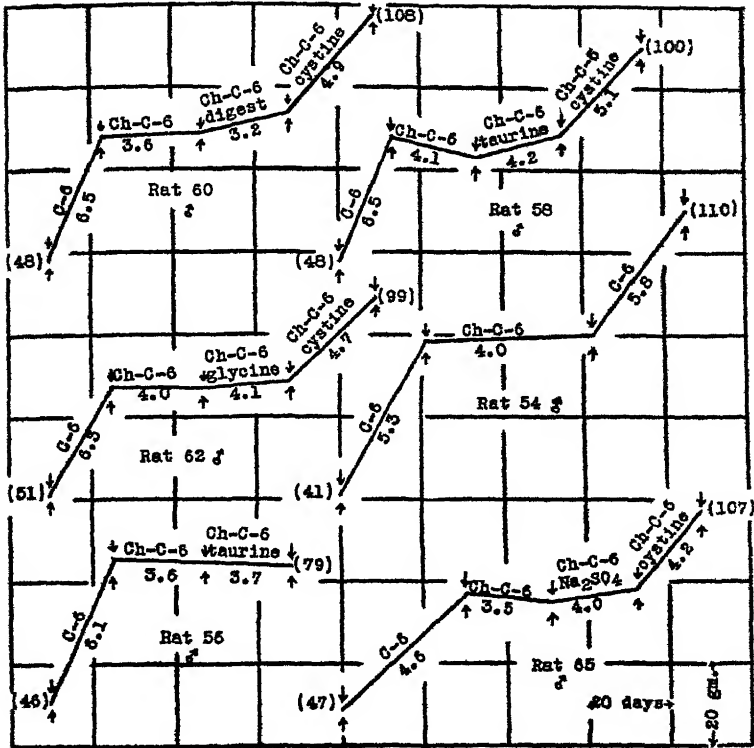


CHART II Growth on cholic acid-basal diet as influenced by the addition of various supplements, and on the basal diet following the removal of cholic acid from the ration. The designations for diets employed, food consumption, and body weights are the same as in Chart I. The taurine curves are chosen from a group of five animals; the curves illustrating the effect of a sodium sulfate, a glycine, or an amino acid digest supplement are representative of groups of five, three, and two animals, respectively; the curve for Rat 54 is typical of that obtained with each of two animals.

#### DISCUSSION

Some toxicity due to the cholic acid feeding was evident in a number of the animals. At autopsy, the liver was frequently



swollen, distinctly pale in color, and somewhat mottled. It is possible that the effect of the cholic acid on growth may be in part related to the toxicity of this compound, but it should be emphasized that stimulation or cessation of growth was exactly coincident with the addition or withdrawal of the extra cystine or methionine. The negative results with taurine are particularly striking and furnish additional evidence that taurine is incapable of replacing cystine for nutritional purposes in the white rat. The results may be considered to support the suggestion by Lewis (2, 3) that the formation of taurocholic acid in the organism occurs subsequent to the conjugation of cholic acid with cystine. The ability of methionine to act as does cystine under the described experimental conditions supplements accumulating data which suggest interesting metabolic interrelationships between these two sulfur-containing amino acids.<sup>9</sup>

The type of experimental procedure used in this investigation was suggested by the studies of White and Jackson (8) who were able to affect the utilization of cystine and methionine, in the growing white rat, by incorporating bromobenzene in the basal diet. In that work it was suggested that the added demand by the organism for the sulfur-containing amino acids for the synthesis of bromophenylmercapturic acid limited the quantities of these amino acids available for growth. In the present investigation, the deficiency in cystine and methionine produced by the cholic acid, and manifested by alterations in growth, may be due to the demand of the organism for taurine for the synthesis of taurocholic acid. It should be pointed out, however, that the results obtained might find possible explanation in a direct detoxication of cholic acid by a mechanism involving either cystine or methionine or both, with the formation of a product other than the bile acid. The negative results with taurine and the complete inability of glycine to alleviate the deficiency imposed by the cholic acid (through a synthesis of glycocholic acid<sup>10</sup>) suggest this explanation. This possibility is under investigation at the present time.

<sup>9</sup> See the review by Lewis (3).

<sup>10</sup> The assumption is made that rat bile contains both glycocholic and taurocholic acids.

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## PHOSPHATASE CONTENT OF BLOOD SERUM AND TISSUES IN THE RAT FOLLOWING ADMINIS- TRATION OF VITAMINS D AND A

BY PAUL D. CRIMM AND J. W. STRAYER

*(From Boehne Tuberculosis Hospital, Evansville, Indiana)*

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Baumgartner, King, and Page (1), on feeding irradiated ergosterol to rabbits, found that the total phosphatase content of bones was diminished. Page and Reside (2) later reported little or no change of phosphatase content after feeding ergosterol. It has been suggested that they were working with an inactive preparation. Kinard and Chanutin (3) found that irradiated ergosterol caused a rise in phosphatase activity in whole rats up to 60 days, but no change in adult animals despite the disturbance in calcium metabolism. Taylor, Weld, Branion, and Kay (4) found that phosphatase in plasma, bone, and kidney of the rat was reduced after irradiated ergosterol. Hansen (5) reported the reduction of phosphatase in serum in four cases of osteogenesis imperfecta following enormous doses of 10,000X.

### EXPERIMENTAL

The method of Bodansky (6) was followed precisely for the determination of serum phosphatase, which is expressed "in units per 100 cc. of serum, a unit of phosphatase activity being defined as equivalent to 1 mg. of P liberated from a sodium glycerophosphate substrate as the phosphate ion during the 1st hour, at pH 8.6 and at 37° " This is a modification of the method of Kuttner and Lichtenstein (7), with corrections for Beer's law, trichloroacetic acid, and glycerophosphate, as calculated by Bodansky (8). The ordinary serum determinations were frequently checked with the method of Benedict and Theis (9).

The tissues were weighed, ground in a mortar, and extracted with distilled water after the method of Kay (10). After the

material stood for 48 hours and was filtered, phosphatase determinations were performed exactly as was done when serum was used. The amount of phosphatase was then calculated from 1 cc. of extract. No difficulty was experienced because of poor matching, color, or turbidity. It is obvious that by this indirect method tissue extracts *are not comparable with the serum findings*, but are only significant when compared with one another.

*Phosphatase Content of Normal Litter Mate Rats of Different Ages*—The pooled blood of groups of normal rats of different ages was obtained from the vena cava and the phosphatase content determined. The rats in each group were litter mates, except the

TABLE I  
*Serum Phosphatase Content of Normal Rats*

Age	No of rats in group	Serum phosphorus per 100 cc	Substrate phosphorus per 100 cc	Phosphatase (by difference)
<i>days</i>		<i>mg</i>	<i>mg</i>	<i>units</i>
42	4	8 50	52 10	43 60
45	7	8 35	46 60	38 25
48	4	8 18	65 10	56 82
55	5	9 40	57 60	48 20
60	5	9 82	56 00	46 12
72	5	8 37	61 00	52 63
150	10	8 73	67 90	58 17
Average	.	.	.	49 11

ten which were 150 days old. Practically no difference existed between the young and old rats as to either phosphorus or phosphatase content of the serum. Bodansky (6) has found human serum to contain approximately 27 units of phosphatase per 100 cc. of blood. Thus, rat serum is about 20 times richer in the enzyme than human serum (Table I).

*Effect of Vitamin D upon Phosphatase Content of Serum and Tissues of Matured Rats*—Ten 150 day-old rats were fed toxic doses of viosterol, 10,000  $\times$ .<sup>1</sup> The drug was administered at the rate of 1 cc. daily per rat for 7 days. At the end of that time the rats

<sup>1</sup> 10,000  $\times$  average cod liver oil; 1,000,000 international units per gm; kindly supplied by Mead Johnson and Company, Evansville

were moribund and were sacrificed. Ten normal controls were also sacrificed (Table II). These rats were the same size and weight,  $\pm 10$  gm. Each determination was made from the pooled tissues of two rats, *i.e.* ten pairs of toxic rats and ten pairs of control rats. The serum calcium in the control rats averaged 11.2 mg. and 16.8 mg. in the rats fed toxic doses of viosterol, 10,000  $\times$ .

TABLE II

*Phosphatase Content of Rat Tissues after Feeding Viosterol, 10,000  $\times$*   
Serum findings were made directly. Other figures represent units of phosphatase per 100 cc. of tissue extract

No. of rats	Serum Ca per 100 cc	Units of phosphatase					
		Blood	Bone	Kidney	Spleen	Liver	Small intestine
	mg						
10 (Controls)	11.2	58.17	57.38	56.36	1.51	0.61	54.85
10 (Poisoned)	16.8	14.48	66.35	14.70	1.74	0.03	150.35

TABLE III

*Phosphatase Content in Rats Deficient in Vitamin A and in Rats Receiving Toxic Dose of Vitamin A Concentrate*

No. of rats, age, and diet	Serum phosphorus per 100 cc	Substrate phosphorus per 100 cc	Phosphatase (by difference)
	mg.	mg	units
5 xerophthalmic rats, 71 days old, 50 days on diet deficient in vitamin A	6.60	30.20	23.60
4 xerophthalmic rats, 85 days old, 64 days deficient	6.92	16.10	9.18
5 toxic rats, approximately 90 days old; each received 584,000 international units vitamin A concentrate during 7 days (very sick)	7.42	23.64	16.22

The reduction of phosphatase activity in blood and kidney is in agreement with that found by Taylor, Weld, Branion, and Kay (4). These authors also report a reduction in bone, but our findings were inconclusive. In four rats (two pairs) the phosphatase content was found to be decreased and in six rats (three pairs) it was increased, the general average showing a decided increase over the control group. Spleen was found to contain very

small amounts of the enzyme and liver almost negligible quantities, both before and after the administration of viosterol. Of particular interest is the great increase of enzyme in the small intestine. The entire small bowel was used, from stomach to cecum. It was split and washed free of its fecal contents with tap water. Except for bone and, of course, liver and spleen, all of the changes in each pair were in the same direction as the average shown in Table II.

*Phosphatase Content of Rat Serum in Vitamin A Deficiency and in Hypervitaminosis A*—It is of interest to note that serum phosphatase is diminished in vitamin A deficiency, hypervitaminosis A, as well as in hypervitaminosis D. The unstable nature of the enzyme is demonstrated in the following experiment. Two groups of rats were used (Table III). In the first group the rats were deficient in vitamin A and in the second they were toxic from massive doses of a vitamin A concentrate. These results indicate that the presence or absence of vitamin A has little or no effect on phosphatase, but that the relatively great reductions are due to some other factors incidental to a greatly impaired nutrition.

#### SUMMARY

1. The serum of rats of various ages was found to contain 49.11 units of phosphatase per 100 cc. of blood. This is approximately 20 times the amount usually found in normal human serum.
2. Rats made toxic with viosterol showed a marked reduction of phosphatase in blood and kidney.
3. Bone possesses large amounts of the enzyme, but the findings after the administration of viosterol were variable.
4. Following massive doses of viosterol the phosphatase content of the small intestine was greatly increased.
5. The administration of toxic doses of viosterol produced no demonstrable effect on the small quantities of phosphatase present in the liver and spleen.
6. Vitamin A-deficient rats and rats fed high doses of vitamin A showed reductions of phosphatase in their respective sera. This is apparently due to the greatly impaired nutrition of the animals.

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## STUDIES OF MULTIVALENT AMINO ACIDS AND PEPTIDES

### VI. THE ACTION OF PROTEOLYTIC ENZYMES ON CERTAIN SYNTHETIC SUBSTRATES

By JESSE P. GREENSTEIN

*(From the Department of Physical Chemistry, Harvard Medical School,  
Boston)*

(Received for publication, September 13, 1935)

One of the most sensitive methods of determining whether a given chemical linkage occurs in substances of biological origin is to treat such substances with enzymes. If the latter are able to attack such a linkage, the possibility that it occurs naturally may be said to be confirmed.<sup>1</sup> So the peptide linkage of the proteins has been completely established by the innumerable enzyme investigations of Fischer, Abderhalden, Waldschmidt-Leitz, Grassmann, Bergmann, and their coworkers.

While the polypeptide structure of the proteins, involving long chains of amino acids bound in amide linkage, has been further established by the x-ray investigations of Astbury (2) and others, the possibility that there may also exist cyclic configurations within the protein molecule has often been advanced (1, 6). The most probable cyclic structure is the diketopiperazine ring, formed by the union of two amino acids with the loss of the elements of water between the two pairs of  $\alpha$ -amino and  $\alpha$ -carboxyl groups. The chief objections to this hypothesis came from the observations by Waldschmidt-Leitz and Schaffner (15) that the neutral diketopiperazines were completely resistant to the action of both animal or plant proteases and peptidases.

The question was again opened by Matsui (12) and Ishiyama

<sup>1</sup> The converse need not necessarily be true, since the failure to attack a given linkage may be due either to lack of the proper enzyme, or else to the possession of certain structural peculiarities by the substrate which render such attack impossible (*cf.* Bergmann (3)).

(11) who found that those anhydrides containing free carboxyl groups were very slowly split by activated trypsin. Further, Shibata (13) and Tazawa (14) indicated that certain anhydrides which they synthesized from arginine ester and from lysine ester were split by pepsin but not by trypsin. The work of the latter authors is rather incompletely described and their method of synthesis should lead rather to the formation of acid amides than to diketopiperazines.

The author has previously described the synthesis of the diketopiperazine, anhydro-*L*-lysyl-*L*-glutamic acid amide hydrochloride by the condensation of the dipeptide ester in methanol-NH<sub>3</sub>. This crystalline anhydride is optically active, and possesses a free  $\epsilon$ -amino group on the lysine residue and a free  $\gamma$  acid amide group on the glutamic residue of the ring.<sup>2</sup> The action of the proteases, pepsin, trypsin, and papain-HCN were tested on this anhydride. Papain-HCN was also incubated with the neutral diketopiperazine, anhydroaminotricarballylic acid tetraamide (8). In no case was there any evidence of hydrolysis observed up to 24 hours.

The dipeptide tetrapole, *L*-lysyl-*L*-glutamic acid, was found by Bergmann, Zervas, Rinke, and Schleich (5) to be somewhat slowly split by fresh swine erepsin. The peptide, *L*-lysyl-*L*-histidine, was more rapidly digested under the same circumstances. In the present communication is detailed the action of the peptidases from autolyzed yeast on these substrates as well as the action of both plant and animal peptidases on the new peptide, glycyl-*DL*- $\alpha$ -aminotricarballylic acid (8). In all cases the potency of the enzyme preparations was tested by the use of known substrates.

#### EXPERIMENTAL

The progress of the enzymic action was followed by the titration method of Willstätter and Waldschmidt-Leitz (17) in 90 per cent alcohol, with thymolphthalein as indicator. For color standard a 0.0025 M solution of cupric chloride in excess NH<sub>3</sub> was employed, as recommended by Grassmann and Heyde (7). It was found advisable in comparing the color of the turbid protein

<sup>2</sup>The optical activity of anhydro-*L*-lysyl-*L*-glutamic acid amide, not given in the previous paper (9), is as follows: 
$$[\alpha]_D^{25} = \frac{-0.35^\circ \times 9.849}{1 \times 1.005 \times 0.1240} = -27.7^\circ.$$

solutions to mix the standard copper solution with a little infusorial earth. A 90 per cent alcoholic KOH solution, 0.025 N, was used, and that aliquot of peptide or anhydride selected which would give in the event of 100 per cent splitting an increase in the alkali titration of 2 cc. The original concentration of the racemic substrates was, of course, double in molarity that of the optically active materials. All reactions were conducted at 30° with the exception of those where papain-HCN was employed, when a temperature of 40° was maintained. Suitable controls were employed.

The pepsin was a Parke, Davis preparation (1:10,000). 10 cc. of the reaction mixture at pH 1.8 contained 100 mg. of egg albumin plus 40 mg. of pepsin. 2 cc. aliquots titrated after 2.5, 6, and 11 hours showed an increase in the alkali titration of 0.45, 0.55, and 0.60 cc. of 0.025 N KOH respectively.

The trypsin was a Fairchild product. 10 cc. of reaction mixture at pH 7.8 contained 100 mg. of gelatin and 40 mg. of the enzyme. 2 cc. aliquots titrated at 2.5, 6, and 11 hours with 0.025 N KOH showed an increase of 0.85, 1.40, and 1.75 cc. respectively.

Papain was activated with HCN for 2 hours at 40° and at pH 5 according to the method of Willstätter and Grassmann (16). In 10 cc. were contained 100 mg. of gelatin plus 8 mg. of papain. 2 cc. portions removed at 2.5 and 6 hours showed an increase of 0.85 and 2.05 cc. of alkali respectively.

Autolyzed yeast was prepared by liquefying a half pound of fresh yeast with 50 cc. of chloroform and then pouring the mass into 200 cc. of water. After 40 hours standing at room temperature, during which time an exactly neutral reaction was maintained by addition of dilute  $\text{NH}_3$ , the suspension was centrifuged. Swine erepsin was prepared by mechanically removing the mucus from the intestines of freshly killed animals, suspending in 3 times the amount of 87 per cent glycerol, and allowing the suspension to stand a few days after addition of toluene. Just before use the extract was diluted with an equal quantity of cold water and centrifuged. Carboxypeptidase was obtained by extracting commercial pancreatin for several hours with 87 per cent glycerol. The extract was then centrifuged, the supernatant solution diluted with an equal amount of cold water, brought to pH 4, and then treated four consecutive times with alumina gel C- $\gamma$ . The residual

solution was then used for the experiments after neutralization and was free of erepsin.

Lysylglutamic acid and lysylhistidine were prepared according to the method of Bergmann, Zervas, and Greenstein (4). The syntheses of glycyl- $\alpha$ -aminotricarballylic acid and the anhydrides

TABLE I  
*Effect of Proteases and Peptidases*

Substrate	Amount in 2 cc	Increase	Hydrolysis	Increase	Hydrolysis	Increase	Hydrolysis
		Yeast autolyate		Intestinal erepsin		Carboxypeptidase	
		cc 0.025 N KOH	per cent	cc 0.025 N KOH	per cent	cc 0.025 N KOH	per cent
Glycylglycine	0.05	0.69(5)*	34	0.83(4)	41		
dl-Leucylglycine	0.10	1.98(5)	98	1.48(3)	74	0(3)	0
dl-Leucylglycylglycine† . . .	0.10	2.72(5)	136	2.04(4)	102		
Chloracetyl-L-tyrosine . . . . .	0.05			0(4)	0	0.74(2)	37
L-Lysyl-L-glutamic acid . .	0.05	1.16(5)	58	0.88(4.5)	44		
L-Lysyl-L-histidine.	0.023	0.76(5)	82				
Glycyl-dl- $\alpha$ -aminotricarballylic acid	0.10	0.83(5)	41	0.41(4)	21	0(4)	0
		Pepsin		Trypsin		Papsin-HCN	
Anhydro-L-lysyl-L-glutamic acid amide . . .	0.05	0(6,24)	0	0(6,24)	0	0(6,24)	0
Anhydro-dl-aminotricarballylic acid tetraamide	0.10					0(6,24)	0

\* The figures in parentheses represent the hours of digestion

† The results of the action of erepsin on the tripeptide will usually yield a hydrolysis above 100 per cent, owing to the simultaneous presence of aminopolypeptidase and dipeptidase.

have previously been reported (8, 9). The enzyme results are given in Table I.

#### DISCUSSION

It is apparent from the results obtained that anhydrolysylglutamic acid amide is completely resistant to pepsin, trypsin, and

papain-HCN. Anhydroaminotricarballylic acid tetraamide is not attacked by papain-HCN. While these results are not sufficient in themselves to discredit the diketopiperazine hypothesis, they do imply that considerable caution should be exercised in applying the anhydride structure to the proteins.

Both lysylglutamic acid and lysylhistidine are split by the peptidases of yeast, the former more slowly than the latter (*cf.* (5) p. 27). The investigations of Greenstein, Wyman, and Cohn (10) have shown that lysylglutamic acid, in contradistinction to other peptides hitherto studied, exists in solution as a rather rigid, rod-like molecule. It may be that this extended form of the molecule renders attack by dipeptidase more difficult. Glycyl- $\alpha$ -aminotricarballylic acid, a peptide differing in structure from glycylglutamic acid by the presence of a carboxyl group on the  $\beta$ -carbon atom, is quite slowly hydrolyzed by yeast and by animal erepsin. This is a phenomenon characteristic of those peptides wherein glycine occupies the acyl position.

Since carboxypeptidase is effective on somewhat acidic substrates, its action on the tricarboxylic peptide was attempted. The results were negative.

#### SUMMARY

1. The action of pepsin, trypsin, and papain-HCN on the diketopiperazine, anhydro-*l*-lysyl-*l*-glutamic acid amide, has been investigated, as well as the action of papain-HCN on anhydro-*dl*-aminotricarballylic acid tetraamide. The results were completely negative.

2. The dipeptides, *l*-lysyl-*l*-glutamic acid and *l*-lysyl-*l*-histidine, are hydrolyzed by yeast peptidase, the former more slowly than the latter. Glycyl-*dl*- $\alpha$ -aminotricarballylic acid is slowly split by yeast and by intestinal erepsin; not at all by carboxypeptidase.

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# THE ADENINE NUCLEOTIDE CONTENT OF HUMAN BLOOD

## II. CORRELATION WITH HEMOGLOBIN

By MARY V. BUELL

*(From the Chemical Division, Department of Medicine, the Johns Hopkins Hospital and the Johns Hopkins University, Baltimore)*

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It was reported recently (1) that the adenine nucleotide content of the blood of normal women was distinctly lower than that of normal men, but that the corpuscular nucleotide concentration was the same for both sexes. In this respect the nucleotide behaves as hemoglobin does. It becomes a matter of interest to investigate any possible correlation between the adenine nucleotide of the erythrocytes and such factors as (1) hemoglobin, (2) volume of packed red cells, and (3) red cell count.

Normal blood does not afford an opportunity for an extensive study of such relationships because of the limited range of variation of these factors in this medium. In the anemias and polycythemias, however, variations are so great as to offer an excellent opportunity for the study of possible correlations. Accordingly, the blood of 100 patients suffering from anemia, a few suffering from polycythemia, as well as 100 normal bloods have been analyzed for nucleotide, hemoglobin, hematocrit, and erythrocyte count. Correlations have been calculated between nucleotide and each of the factors mentioned with the result that a linear relationship, maintained over 10-fold variations in concentrations, has been demonstrated between nucleotide and hemoglobin.

### EXPERIMENTAL

*Cases Studied*—The normal bloods were drawn by venepuncture from medical students, nurses, and laboratory workers into bottles containing the correct amount of oxalate. The pathological cases studied were patients at the Johns Hopkins Hospital. The



anemias comprised an extraordinary variety of cases both morphologically and etiologically. The red cells were in some cases macrocytic, in others microcytic, and in still others normocytic. Hypochromic, hyperchromic, and sickled cells were included. The anemias were caused by acute or chronic blood loss, excessive blood destruction, a variety of toxemias, including those of pregnancy, and also certain aplastic and idiopathic anemias. The cases were either untreated or in various stages of treatment, certain individuals being followed at intervals during treatment. Those cases were excluded which were associated with abnormal metabolic conditions such as diabetes, leucemia, carcinoma, nephritis, etc. Otherwise no selection of data has been made.

### *Methods*

The adenine nucleotide was determined in duplicate by the Buell (1) method. Hematocrits were read in especially calibrated Wintrobe tubes after rapid centrifugation for 30 minutes. Duplicate red cell counts and hemoglobin determinations were painstakingly performed by Miss Regina Weistock. Statistical treatment of the data was carried out under the direction of Dr. C. E. Palmer of the Johns Hopkins School of Hygiene and Public Health.

### *Results*

The data obtained have been plotted in Charts I to III, which illustrate the correlation between nucleotide and (1) hemoglobin, (2) hematocrit, and (3) erythrocyte count, respectively. In all three charts the ordinates are identical and represent the mg of adenine nucleotide per 100 cc. of whole blood. In each case the unit on the abscissa has been so chosen as to make the curves as nearly as possible directly comparable. The circles represent normal bloods and the crosses pathological bloods.

In Chart I the abscissa represents gm. of hemoglobin per 100 cc. of whole blood. It is evident that there is a definite spread among the points representing normal bloods which defines the limits of normal variation. When parallel lines, drawn to outline these normal limits of variation, were extended in each direction to include the pathological bloods, it became apparent that these points also fell within the same lines. In other words, there was no greater actual deviation in the relationship between nucleotide

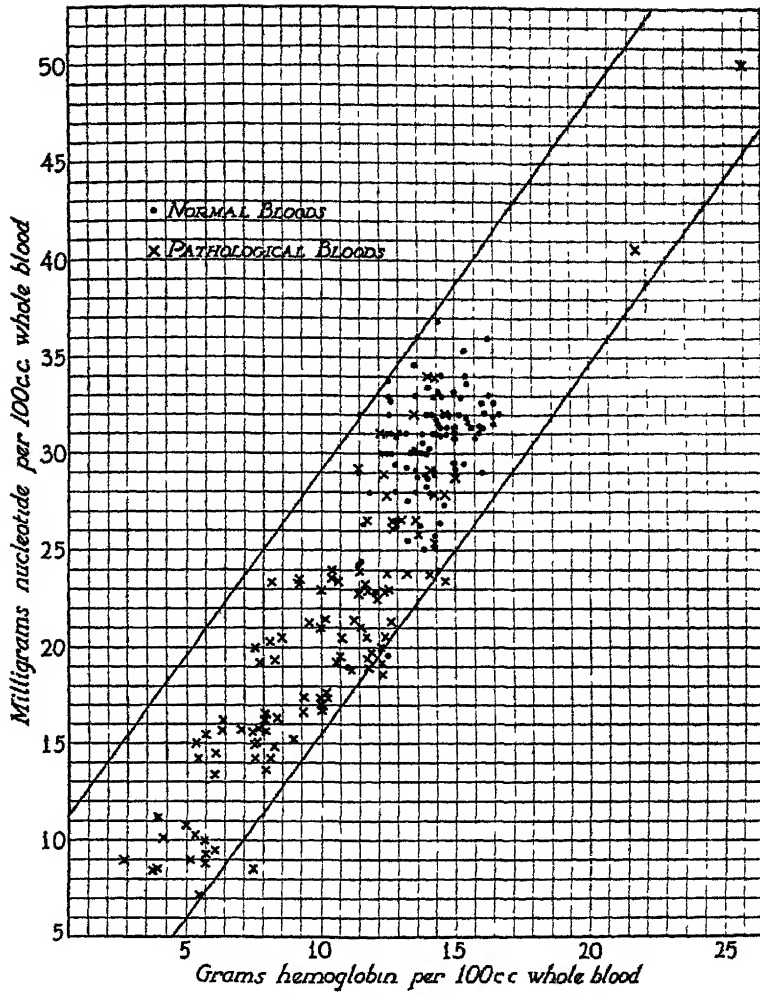


CHART I The correlation between nucleotide and hemoglobin; the coefficient was found to be +0.898.

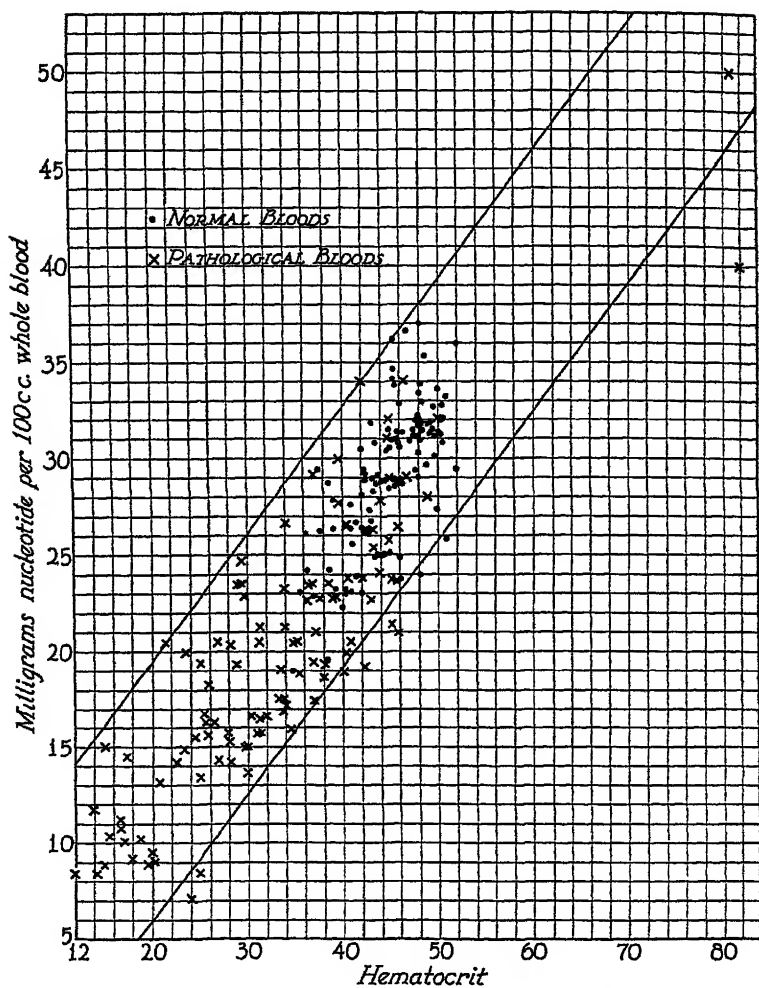


CHART II The correlation between nucleotide and hematocrit; the coefficient was found to be  $+0.888$ .

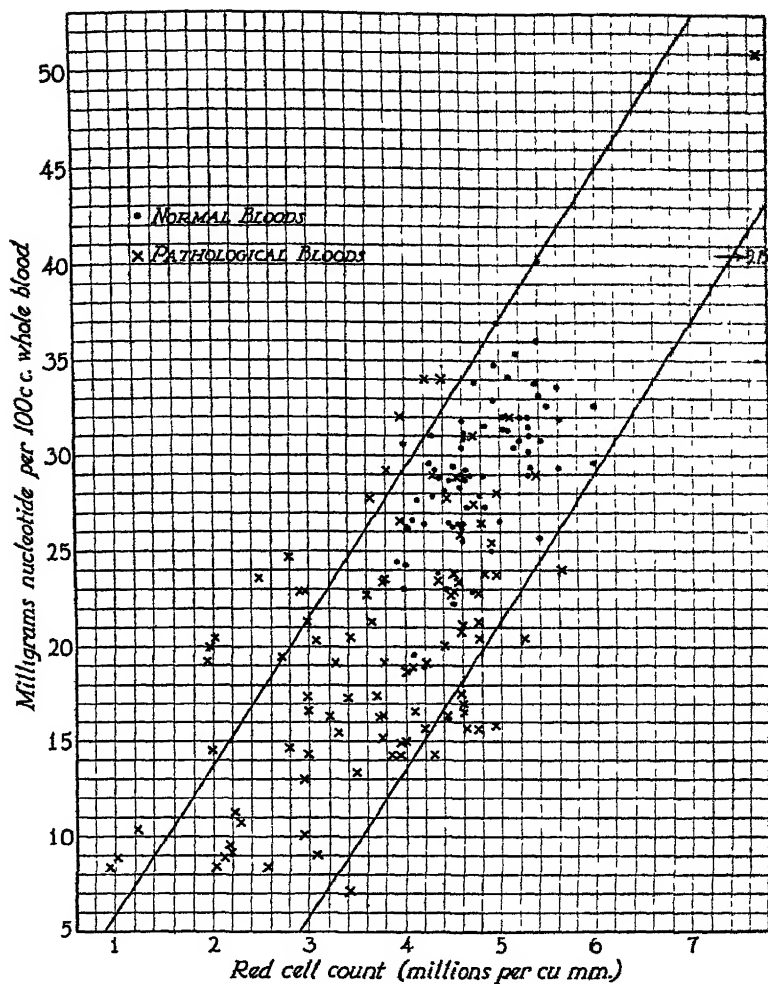


CHART III. The correlation between nucleotide and erythrocyte count; the coefficient was found to be  $+0.723$ .

and hemoglobin among the pathological bloods than among the normal. Over this extraordinary range of hemoglobin concentration (2.5 to 25 gm. per 100 cc. of blood) there was an approximately linear relation.<sup>1</sup> The correlation coefficient, calculated to be  $+0.898$ , demonstrates an unequivocal correlation between nucleotide and hemoglobin.

Chart II illustrates the relation between nucleotide and hematocrit, *i.e.* the volume of packed red cells. Here again, the correlation is good, the coefficient being  $+0.888$ . It will be noted, however, that one of the polycythemias which fell accurately into line in Chart I fell distinctly out of line in Chart II. Chart III shows the relation between nucleotide and erythrocyte count. For the normal bloods the same limits of variation were found as in Charts I and II but, by contrast, the points representing the pathological bloods were more scattered. The same case of polycythemia previously referred to, which was somewhat out of line in Chart II, could not be plotted on Chart III and should be located at a point corresponding to 9.15 on the abscissa. Nevertheless, it is evident that there is a certain correlation between nucleotide and erythrocyte count, the coefficient being  $+0.723$ . The possibility of some correlation between nucleotide and red count was vaguely suggested by Rothmann (2).

#### DISCUSSION

In the interpretation of these observations consideration must be given to the factors, both technical and physiological, which influence the values. Among the physiological may be mentioned (1) the rates of synthesis, destruction and excretion of the end-products of the nucleotide, (2) variable proportions of leucocytes, which contain relatively more nucleotide than erythrocytes, and (3) the possible variable occurrence of other purine nucleotides such as oxyadenine nucleotide. These, among other factors, doubtless contribute to the variations observed among normal bloods.

In any exact comparison of the correlation coefficients noted

<sup>1</sup> On Chart I it is impossible to distinguish between the successfully treated anemias and polycythemias; it would be impossible to distinguish such bloods from strictly normal bloods if they were not marked with dots and crosses.

above allowance must also be made for technical variations in the reproducibility of the determinations involved. The error inher-

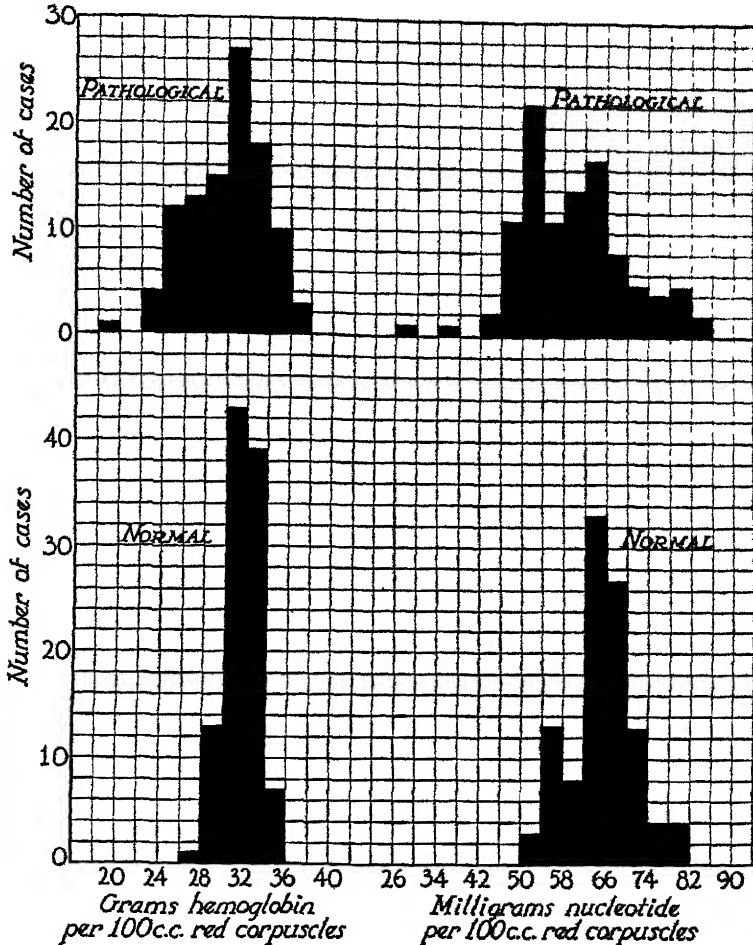


CHART IV. There is a general similarity between the frequency distribution patterns of nucleotide and hemoglobin in both the normal and pathological bloods studied.

ent in the nucleotide determinations is the same in Charts I to III. Although the errors expected in the three determinations

with which the nucleotide has been correlated cannot be stated mathematically with assurance, it can be said confidently that the greatest degree of accuracy was obtained in the hematocrit and the least in the hemoglobin determinations. Actually the apparent correlation of nucleotide was best with hemoglobin, but not significantly better than with hematocrit. This small observed superiority would doubtless have appeared greater had the same degree of precision been attained in the determination of hemoglobin and hematocrit.

Chart IV illustrates the frequency distribution of nucleotide and hemoglobin in both the normal and the pathological bloods studied. On the ordinates is given the number of cases studied; on the abscissæ the gm. of hemoglobin and the mg. of adenine nucleotide per 100 cc. of erythrocytes. It will be seen that many of the pathological bloods were hypochromic; *i e.*, the mean corpuscular hemoglobin concentration was low. Similarly there has been a change in the distribution pattern of nucleotide, which follows, in a general way, that of hemoglobin.

#### SUMMARY

There is in human blood a significant correlation between the adenine nucleotide and hemoglobin contents of the erythrocytes. If this correlation indicates a primary association between nucleotide and hemoglobin, the observed correlation between nucleotide and both hematocrit and erythrocyte count follows logically. It cannot be concluded from these observations, however, whether both compounds are regulated by the same unknown factors, or whether the occurrence of one fundamentally affects the other.

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## THE ISOLATION OF PECTIC SUBSTANCES FROM WOOD\*

By ERNEST ANDERSON

*(From the Carnegie Institution of Washington, Division of Plant Biology, Stanford University, California, and the Department of Chemistry, the University of Arizona, Tucson)*

(Received for publication, September 27, 1935)

Pectin is considered by many investigators to be the tetramethyl ester of an acid composed of 4 molecules of *d*-galacturonic acid, 1 molecule of *d*-galactose, and 1 molecule of *L*-arabino-*e* (1). However, experimental work described by Morell, Baur, and Link (2) indicates that the pectin molecule is more complex and contains between 8 and 10 molecules of *d*-galacturonic acid. The data obtained by repeatedly crystallizing calcium pectate and analyzing the various crops support the view of Link and his collaborators. Pectic substances readily lose a part of their combined sugar during purification. As a result, the data obtained on analysis of a pectate will depend on the previous treatment of the material.

Several explanations have been given for the origin of pectic substances, hemicelluloses, and lignin in plants. Candlin and Schryver (3) suggested that the hemicelluloses are formed from pectic substances. Other workers believe that lignin is formed from pectin. Buston (4) has discussed the relation among these classes of compounds. He concludes that they are not formed from each other. The present investigation supports the conclusions of Buston. It is possible that pectic substances, hemicelluloses, lignin, and perhaps some phenolic bodies are formed from carbohydrates by a process of intermolecular oxidation and reduction whereby energy is liberated for use by the growing plant.

\* The author desires to express his appreciation to the National Research Council for assistance in this investigation. The results of this investigation were described by the author at the meeting of the Pacific Coast Division of the American Association for the Advancement of Science at Berkeley, June, 1934, and at the meeting of the American Chemical Society at San Francisco, August, 1935.



The possible presence of pectic materials in mature wood is a debated question (5). Most workers agree that they are present in young wood but claim that they are absent from mature wood. However, Preece (6) found 0.4 per cent pectin in boxwood and O'Dwyer (7) isolated a pectic material from beech wood. Experimental work<sup>1</sup> extending over the past 3 years has led to the isolation of pectic substances from the cambium layer and from the sap-wood of black locust, *Robinia pseudacacia*, L. While the work on the heart-wood of black locust is incomplete, pectic materials seem to be present in it also. The investigation indicates that the pectic substances and hemicelluloses remain in wood very much as they were originally laid down. Other substances seem gradually to be deposited on the original materials and protect them from the action of solvents. After the covering substances have been removed, the pectic materials can be dissolved by use of suitable solvents. Sands and Nutter (9) found that treatment of a wood with chlorine and ammonium hydroxide for the removal of lignin renders soluble large amounts of hemicellulose not previously dissolved by 5 per cent sodium hydroxide solution. The failures of many investigators to isolate pectic substances from wood are probably due to the protective action of these covering substances.

Pectic materials are present in plants in several forms of combination (1). The water-soluble pectic material extracted from wood is present partially as a methyl ester. The water-insoluble pectic material in wood appears to be present chiefly as the calcium salt of pectic acid, though some of it may be combined with cellulose or lignin. Several carboxyl groups are present in a molecule of pectic acid. Each of these may be combined differently. One may be present as a methyl ester, another as a calcium salt, while a third may be combined with cellulose or lignin. For the removal of water-insoluble pectic substances the wood is first heated with ammonium oxalate solution or dilute hydrochloric acid and then treated with ammonium hydroxide. This procedure would indicate that the pectic material is present as calcium

<sup>1</sup> This work has been done in cooperation with Dr. I. W. Bailey. The work of Bailey and Kerr (8) made possible the selection of suitable methods for isolating the various polyuronides from wood and for determining the location of each polyuronide in the cell

pectate. Onslow (10) suggests that the pectic material isolated by O'Dwyer (7) from beech wood was present as calcium pectate. The work of Sucharipa (11) indicates that pectic substances are sometimes combined with cellulose. In the present investigation an attempt was made to dissolve all the cellulose from wood by the use of Schweitzer's reagent and leave the pectic material undissolved. This was not accomplished. Considerable cellulose remained undissolved. This might indicate a union between pectic material and cellulose. On the other hand the undissolved cellulose may have been surrounded by insoluble pectic material instead of being present as a compound of cellulose and pectin.

The work of Bailey and Kerr (8) indicates that the pectic materials remaining in wood after thorough extraction with boiling water are present in the intercellular layer (middle lamella) and in the primary cell wall. The latter also contains large amounts of cellulose. Later, as the thick secondary cell wall forms inside the primary cell wall, large amounts of cellulose and hemicellulose are deposited in it. Lignin is deposited along with and over these materials or possibly in part combined with them. The fact that the cambium layer gives much larger amounts of carbon dioxide than the sap-wood, when heated with 12 per cent hydrochloric acid, would be readily explicable if the pectic materials are located in the middle lamella and the primary cell wall.

#### EXPERIMENTAL

*Material Used*—Black locust wood, *Robinia pseudacacia*, L., was chosen for investigation because it is not heavily lignified. It was collected by Dr. I. W. Bailey near Boston, in April, 1933. At the time of cutting it was separated into three portions; namely, the cambium-phloem, sap-wood, and heart-wood. Each portion was air-dried and converted to a fine powder.

*Uronic Acid Anhydride Present*—Treatment of the wood with 12 per cent hydrochloric acid by the method of Lefèvre and Tollens (12) gave the following results: cambium 2.4, sap-wood 1.12, and heart-wood 1.09 per cent carbon dioxide. These results would correspond respectively to the following amounts of uronic acid anhydride: 9.60, 4.48, and 4.36 per cent. The slight decrease in the amount of carbon dioxide given by the heart-wood is undoubtedly due to the later deposition of other materials in the

wood. The author has heated the sap-wood and heart-wood of several different species of trees with 12 per cent hydrochloric acid. In all cases the yield of carbon dioxide given by the sap-wood was slightly higher than that given by the heart-wood.

### *Sap-Wood*

*Isolation of Pectic Materials*—The powdered wood was thoroughly extracted by ether and by hot alcohol. It was then extracted with hot water until the starch had been removed. It was next extracted twice with a 5 per cent solution of sodium hydroxide, each time for 48 hours. The alkaline extracts contained the hemicellulose, which will be described in a later article. This procedure removed some materials that were covering the pectic substances and preventing their solution by the pectic solvents. The wood was now washed with water and with very dilute hydrochloric acid to neutralize the alkali. It was then extracted twice with 0.05 N hydrochloric acid. Each time it was heated in a bath of boiling water for 2 hours. This process apparently removed the calcium from calcium pectate and left most of the pectic acid to be dissolved by ammonium hydroxide. At the same time some pectic acid was dissolved by the hot hydrochloric acid. The combined hydrochloric acid extracts were made slightly alkaline with ammonium hydroxide, then slightly acid with acetic acid, and a 10 per cent solution of calcium chloride was added.<sup>2</sup> Pectin A was precipitated by addition of 3 volumes of alcohol. The supernatant liquid was siphoned off and the precipitate centrifuged out and washed. The yield was 0.8 per cent of the weight of the wood used. Samples of Pectin A, prepared at different times, when heated with 12 per cent hydrochloric acid, gave amounts of carbon dioxide varying from 5 to 11 per cent. The wood was next extracted twice, each time for 48 hours, with a 5 per cent solution of ammonium hydroxide. The combined ammonium hydroxide extracts were made acid with acetic acid and Pectin B precipitated as described above. The yield was 0.7 per cent.

*Purification of Pectic Substances*—A portion of Pectin A was shaken with 150 times its weight of 1 per cent hydrochloric acid and the solution centrifuged from the solid impurities. The solu-

<sup>2</sup> Branfoot (1) describes the determination of pectic materials

tion was made alkaline with clear barium hydroxide solution and then slightly acid with acetic acid. The precipitate of barium pectate was centrifuged out and washed several times. On analysis it gave 16.4 per cent carbon dioxide by the method of Lefèvre and Tollens (12), 22.5 per cent barium, and 14.05 per cent furfural. Barium pectate prepared from commercial citrus pectin by the same method gave 16.3 per cent carbon dioxide, 23 per cent barium, and 15 per cent furfural.

A second portion of Pectin A was shaken with 0.1 N hydrochloric acid and centrifuged from the insoluble material. The pectic material was precipitated by addition of 3 volumes of alcohol and the precipitate centrifuged out. The dry pectic material was dissolved in 20 parts of warm 0.1 N sodium hydroxide solution and allowed to stand for 1 hour. The solution was acidified with hydrochloric acid, then made alkaline with ammonium hydroxide, and filtered. The filtrate was made faintly acid with acetic acid and a 10 per cent solution of calcium chloride was added. The calcium pectate was filtered from the hot solution and washed with boiling water until free of chlorides. On analysis it gave 16.5 per cent carbon dioxide, 7.8 per cent calcium, 27 per cent furfural, and 42 per cent mucic acid. Calcium pectate prepared from commercial citrus pectin by the above method gave 19.10 per cent carbon dioxide, 8.6 per cent calcium, 19.5 per cent furfural, and 51 per cent mucic acid. It appears that calcium pectate made from Pectin A contains a larger amount of pentose than that from commercial citrus pectin. However, when this calcium pectate is redissolved and reprecipitated several times, the amount of pentose gradually decreases and its composition approaches closely calcium pectate made from citrus pectin. For example, after three such treatments the calcium pectate made from Pectin A gave 18.8 per cent carbon dioxide, 8.80 per cent calcium, and 18.8 per cent furfural.

Pectin B was mixed with 150 parts of water and allowed to stand for 24 hours. After centrifuging, the clear solution was made 1 per cent acid with hydrochloric acid and the pectic material precipitated with alcohol. The precipitate was centrifuged out and washed. On analysis it gave 22.3 per cent carbon dioxide, 1.00 per cent ash, and 2.32 per cent methoxyl. Link and collaborators have shown that the polygalacturonic acid from commercial

citrus pectin consists of between 8 and 10 molecules of *d*-galacturonic acid. A compound of 8 molecules of *d*-galacturonic acid and 1 molecule of *l*-arabinose with one carboxyl group present as a methyl ester should give 22.3 per cent carbon dioxide and 2 per cent methoxyl. It thus appears that Pectin B is similar in composition to the polygalacturonic acid from commercial citrus pectin.

Purified Pectin B was converted to calcium pectate by the procedure described under Pectin A. The resulting calcium pectate gave 18.9 per cent carbon dioxide, 9.0 per cent calcium, 18 per cent furfural, and 50 per cent mucic acid.

*Identification of the Uronic Acid*—The presence of large amounts of galacturonic acid in the pectic material was established by the method of Heidelberger and Goebel (13). At different times purified pectic materials as well as pure calcium pectate from sapwood were mixed with hydrobromic acid and bromine and heated under a reflux. In all such cases mucic acid melting at 217° was isolated from the solution.

The calcium pectate prepared from Pectins A and B was dissolved in 4 per cent sulfuric acid and the solution heated to boiling for 25 hours. The solution was filtered and neutralized with calcium carbonate. Eventually there were obtained a white, water-soluble, calcium salt and an alcohol solution containing small amounts of sugar. The calcium salt, on analysis, gave 20.5 per cent carbon dioxide, 10.1 per cent calcium, and 18.6 per cent furfural. It showed  $[\alpha]_D^{25} = +48.7^\circ$ . When oxidized with bromine water it gave large amounts of mucic acid melting at 217°. This establishes the presence of *d*-galacturonic acid.

The amount of sugar obtained from the hydrolysis of the calcium salt was so small that it could not be identified. A pentose sugar is present in the purified pectic material first obtained. However, in the process of conversion to the purified calcium salt most of the sugar was liberated. No methyl pentose seems to be present at any time, since only traces of the furfural phloroglucide precipitate dissolve in hot alcohol.

### *Cambium*

*Isolation of Pectic Substances*—The powdered cambium was extracted twice with acetone and twice with boiling alcohol. It

was then extracted three times with water at 97°, each time for a period of 3 hours. The combined water extracts were mixed with a 10 per cent solution of calcium chloride and 3 volumes of alcohol added. The yield of Pectin A was 6 per cent of the weight of the wood used. The wood was next extracted twice with 0.05 N hydrochloric acid. Each time it was heated for 2 hours in a bath of boiling water. Pectin B was isolated from the hydrochloric acid extract as described above. The yield was 5.6 per cent. The wood was next extracted twice, each time for 24 hours, with a 5 per cent solution of ammonium hydroxide. The combined filtrates were acidified with acetic acid and Pectin C was precipitated with calcium chloride and alcohol. The yield was 2 per cent.

Pectins A and B gave strong tests for starch but Pectin C was starch-free. The three lots of pectin gave respectively the following amounts of carbon dioxide by the method of Lefèvre and Tollens (12): 8.5, 11.0, and 9.5 per cent. All three of the pectins gave positive tests for ester-linked methoxyl groups but negative tests for ether-linked methoxyl groups.

*Purification of Pectic Materials*—Pectins A and B were mixed separately with 50 times their weight of 1 per cent hydrochloric acid and heated in a bath of water at 85° until the test for starch was negative. This required approximately 3 hours. The solutions were filtered and the pectic material precipitated with alcohol. Pectin A gave 16.3 per cent carbon dioxide, 2.75 per cent methoxyl, and 16 per cent furfural. Pectin B gave 16.6 per cent carbon dioxide, 4.7 per cent methoxyl, and 15 per cent furfural. Monomethyl pectinic acid, corresponding to the formula of Nanji, Paton, and Ling (14), should give 17.4 per cent carbon dioxide, 3.06 per cent methoxyl, and approximately 20 per cent furfural.

*Conversion of Pectic Materials to Calcium Pectate*—Purified Pectins A, B, and C were dissolved separately in 3 per cent ammonium hydroxide and the solutions centrifuged. The clear solutions were mixed with 1 per cent of their weight of sodium hydroxide and allowed to stand for 1 hour to hydrolyze the ester. They were then made 1 per cent acid with hydrochloric acid and the pectic acid precipitated with alcohol. In each case the pectic acid was centrifuged out and washed with alcohol. It was dissolved in dilute ammonium hydroxide and the clear solution made

slightly acid with acetic acid. The calcium pectate was precipitated from the hot solution by addition of a 10 per cent solution of calcium chloride. The calcium pectate was filtered off and washed with boiling water until free of chlorides. The three lots of calcium pectate gave the following results on analysis: Pectate A, 19.3 per cent carbon dioxide, 9.0 per cent calcium, 32.6 per cent furfural, 45 per cent mucic acid; Pectate B, 19.0 per cent carbon dioxide, 8.6 per cent calcium, 20.5 per cent furfural; Pectate C, 18.9 per cent carbon dioxide, 9.0 per cent calcium, 18.75 per cent furfural. The results obtained on analysis of calcium pectate made from commercial citrus pectin have already been given. The properties of these calcium pectates were similar to those of known samples of calcium pectate. The presence of *d*-galacturonic acid in them was established by the method used in the case of the sap-wood.

*Amount of Pectic Material in Cambium and Sap-Wood*—The method used to determine the amount of pectic material in soft vegetable tissues (1) cannot be applied to wood because of the insolubility of pectic material in wood. However, from the amount of carbon dioxide obtained by heating the wood with 12 per cent hydrochloric acid, an estimate can be made of the maximum possible amount of pectic material present. In the case of the cambium this is approximately 13 per cent. The amount of pectic material in the cambium is undoubtedly much less than 13 per cent. If one-half of the carbon dioxide given by the sap-wood of black locust when heated with 12 per cent hydrochloric acid came from pectin, there should be between 3 and 4 per cent of pectic material in the sap-wood. Probably the amount actually present in the sap-wood is less than 3 per cent.

#### SUMMARY

Pectic substances have been isolated from the cambium layer and from the sap-wood of black locust. The composition of some of these materials has been found to approximate closely certain of the pectinic acids. Others of the pectic materials were found to approximate closely the polygalacturonic acid obtained from commercial citrus pectin. These materials were converted to calcium pectate and its composition found to approximate closely calcium pectate made from citrus pectin. The presence of *d*-

galacturonic acid was established in the calcium pectate from both cambium and sap-wood. The sugars were not identified but methyl pentose sugars are apparently absent. It appears that the pectic materials in wood are deposited in the middle lamella and the primary cell wall in the early stages of cell development and remain even in the old wood. Later in the growth of the wood other materials appear to be deposited on the pectic materials and protect them from the action of pectic solvents. The water-insoluble pectic material seems to be present largely as a calcium salt, though a part may be combined with cellulose or with lignin. While no determination was made of the amount of pectic material present, the sap-wood apparently contains less than 3 per cent and the cambium less than 13 per cent.

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## THE CHEMISTRY OF THE LIPIDS OF YEAST

### III. LECITHIN AND CEPHALIN\*

By L. F. SALISBURY† AND R. J. ANDERSON

*(From the Department of Chemistry, Yale University, New Haven)*

(Received for publication, October 10, 1935)

In the preceding papers in this series Newman and Anderson (1) reviewed the small amount of work which had been published on the phospholipids of yeast and they also reported the isolation of about 1.3 per cent of crude phospholipids, calculated on the weight of the dried yeast. The mixed phospholipids were purified and the hydrolysis products were examined but no attempt was made at that time to separate the mixture of lecithin and cephalin into pure components. The proportion of lecithin and cephalin in the purified phospholipid was estimated by the ratio, total N to amino N, and was as 4:1. In the examination of the unsaturated fatty acids the authors mentioned above found that catalytic hydrogenation (2) yielded a mixture of palmitic and stearic acids, thus indicating the presence of both  $C_{16}$  and  $C_{18}$  unsaturated acids.

The present report deals with the separation of the purified mixed phospholipids into lecithin and cephalin and with the detailed investigation of the cleavage products obtained on hydrolysis. One of the problems in the present investigation was to determine whether the unsaturated  $C_{16}$  acid formed a part of the lecithin or of the cephalin molecule. As a matter of fact, it was found that both lecithin and cephalin contained the unsaturated  $C_{16}$  acid; the lecithin, however, contained the larger amount.

As judged by the iodine number of the mixed unsaturated acids, no acid having more than one double bond was present. This

\* The data are taken from the dissertation submitted by L. F. Salisbury to the Faculty of the Graduate School, Yale University, 1935, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

† Holder of the Fleischmann Fellowship in Chemistry, Yale University, 1933-35.

was further confirmed by an experiment in which the mixed unsaturated acids were brominated but no insoluble bromides were obtained.

The purified lecithin, upon hydrolysis, yielded 64 per cent of fatty acids of which 14 per cent were solid and 86 per cent were liquid acids. The solid acids contained approximately 43 per cent palmitic and 57 per cent stearic acid. The liquid acids were wholly of the  $C_nH_{2n-2}O_2$  type, and no liquid saturated acids were present. Upon reduction of the liquid acids, 63 per cent palmitic and 37 per cent stearic acid were found; a trace of some acid higher than stearic acid was indicated. From the aqueous portion of the hydrolysis mixture choline, optically active glycerophosphoric acid, and phosphoric acid were isolated.

Purified cephalin yielded, upon hydrolysis, 64 per cent of fatty acids, 16 per cent solid, and 84 per cent liquid acids. The solid acids contained 56 per cent stearic and 44 per cent palmitic acid. The liquid acids were reduced and found to be free of liquid saturated acids. The reduced acids contained 58 per cent stearic and 42 per cent palmitic acid, a trace of an acid lower than palmitic was possibly indicated. From the aqueous portion of the hydrolysis mixture aminoethyl alcohol, inactive glycerophosphoric acid, and phosphoric acid were isolated.

#### EXPERIMENTAL

Through the courtesy of The Fleischmann Laboratories, New York, we were supplied with about 78 kilos of fresh living compressed yeast, *Saccharomyces cerevisiae*, which had been grown under carefully controlled conditions so as to exclude the introduction of extraneous impurities, such as the hydrocarbons found in a former experiment. The yeast was extracted in four equal portions with alcohol and with alcohol and ether and the lipids were isolated as described by Newman and Anderson (1). The same precautions were observed to exclude air by the use of carbon dioxide or nitrogen as described in the publication mentioned above.

The total ether-soluble lipids amounted to 531 gm. and the dried defatted yeast cells weighed 17.29 kilos. The phosphatides were separated from the fat fraction by precipitation with acetone from ethereal solution and purified as described in the former

publication (1). The mixed phosphatides amounted to 58 per cent of the total fat.

The ether-acetone mother liquors were concentrated and cooled, whereupon a considerable quantity of solid material was precipitated. The latter after being purified by crystallization from ethyl acetate was found to consist of nearly pure ergosterol, m.p. 161–162°,  $[\alpha]_D^{25}$  in chloroform =  $-126^\circ$ . The acetone-soluble fat obtained on evaporation of the acetone solution was not further examined.

#### *Purification of Cephalin*

The mixed phosphatides were triturated with two portions of absolute alcohol, after which the alcohol-insoluble portion was precipitated six times from ethereal solution with alcohol and twice with acetone. A slight amount of ether-insoluble material was separated by sedimentation. The ethereal solution of the crude cephalin was next emulsified with 10 per cent aqueous acetic acid and the mixture was separated by pouring into acetone. The precipitate was collected, dissolved in ether, and all of the operations mentioned above were repeated a second time, after which the cephalin was precipitated three times from ethereal solution with alcohol. The final product, a light brown hygroscopic powder, 32.7 gm, was preserved under absolute alcohol and carbon dioxide.

The following values were found on analysis: C 59.78, H 9.37, N 1.46, P 3.81, amino N 1.50, ash 9.40, iodine number 39.4,  $[\alpha]_D^{21}$  in chloroform =  $+3.6^\circ$ . The potassium hydroxide equivalent of the phosphorus was determined according to Rudy and Page (3) and was found to be 1.16.

The values for carbon, hydrogen, nitrogen, and phosphorus are lower than required by theory for a typical cephalin but are similar to those reported by other investigators. Levene and West (4) have suggested that the low values obtained on analysis are due to partial decomposition of cephalin during purification.

*Hydrocephalin*—It was stated by Levene and West (4) that cephalin was hydrogenated with difficulty and our experiments fully confirm this observation. It was necessary to shake an ethereal solution of cephalin with platinum oxide under 25 pounds pressure of hydrogen for 96 hours before a saturated compound

was obtained. The hydrocephalin was quite soluble in moist chloroform and it dissolved slowly in moist ether but in the dried solvents it was insoluble. For purification the substance was dissolved in moist chloroform and precipitated by the addition of acetone as a white amorphous solid. Found: C 58.43, H 9.41, N 1.97, P 3.71, iodine number 0.9.

*Purification of Lecithin*—From the alcohol-soluble fractions obtained in the purification of cephalin lecithin was separated by the cadmium chloride method and purified as described by Levene and Rolf (5). After the cadmium chloride treatment was repeated a second time, the lecithin was practically free from amino nitrogen. The purified and dried lecithin was a firm plastic mass of light yellowish color. Found: C 62.35, H 10.77, N 2.06, P 4.47, amino N 0.1, ash 2.7, iodine number 37.5,  $[\alpha]_D^{25}$  in chloroform =  $+6.8^\circ$ .

*Ninhydrin Reaction*—Cephalin, which contains amino nitrogen, reacts with ninhydrin to give a blue color. This reaction was used in the purification of lecithin as a qualitative test for the presence of cephalin. Experiments showed that as little as 0.7 mg. of cephalin emulsified in 5 cc. of water to which were added 0.01 cc. of pyridine and 2 drops of a saturated aqueous solution of triketohydrindene gave a distinct blue color within 20 minutes when heated in a boiling water bath. The purified lecithin gave no coloration when tested in this manner. Although the reaction is not very sensitive, it may serve as a qualitative test for cephalin in the separation of mixed phosphatides.

*Hydrolecithin*—Lecithin was easily hydrogenated. An ether-alcohol solution of 6 gm. of lecithin was shaken with platinum oxide under 25 pounds pressure of hydrogen for 1 hour. The catalyst was filtered off and the reaction product was obtained as a white amorphous solid weighing 5 gm. after the solution had been concentrated and cooled. The substance was dissolved in warm ether, treated with norit, filtered, and the solution was cooled. The white amorphous precipitate which separated was filtered off, washed with cold ether, and dried. The substance was slightly soluble in acetone and petroleum ether, easily soluble in hot ethyl acetate, alcohol, and in warm ether, and very soluble in chloroform. On cooling the solutions, only white amorphous precipitates were obtained. The substance softened at  $78^\circ$  and

melted at 92° to a clear fluid;  $[\alpha]_D^{21}$  in chloroform = +7°. The hydrolecithin absorbed no iodine when tested with the Hanus solution. Found: C 62.26, H 11.44, N 1.94, P 4.35.

*Cleavage Products of Lecithin*—The purified lecithin, 22.44 gm., was rubbed into an emulsion with 350 cc. of water which had been boiled, cooled, and saturated with carbon dioxide, and 15 cc. of concentrated sulfuric acid were added. The mixture was refluxed for 6 hours, until the coagulum which formed on acidification had been converted into a clear oily layer. The fatty acids were extracted with ether and the aqueous solution was saved for the examination of water-soluble compounds.

*Separation of Fatty Acids*—The fatty acids, after being dried *in vacuo*, formed a brown oil weighing 14.39 gm. or about 64 per cent of the lecithin. The acids were separated by the lead soap-ether method (6) into 12.03 gm. of liquid acids, having an iodine number of 84.3, and 1.95 gm. of solid acids. The liquid acids amounted to 86 per cent and the solid acids to 14 per cent of the total acids recovered.

*Procedure for Examination of Fatty Acids*—The fatty acids were converted into methyl esters and the esters were fractionated by distillation at 2 to 3.5 mm. pressure. The melting point and refractive index, at 55°, of the esters were taken as criteria of purity. The purified esters were saponified and the acids were isolated and recrystallized. The acids were identified by melting point, mixed melting point, combustion, and molecular weight determination. From a consideration of the indices of refraction and the amounts of the ester fractions, the percentage composition of the esters was estimated.

*Liquid Fatty Acids*—The liquid fatty acids were hydrogenated catalytically (2) and the solid reduced acids thus obtained were methylated. No liquid saturated fatty acids were found. The methyl esters were fractionated, yielding methyl palmitate and methyl stearate. The first residue in the distilling flask was found to contain a trace of some acid of higher molecular weight than stearic acid.

The methyl palmitate melted at 28°;  $n_D^{55} = 1.4263$ . The free acid obtained on saponification melted at 62.5°.

$C_{16}H_{32}O_2$ (256).	Calculated	C 75.00, H 12.50
	Found.	" 74.74, " 12.80
	"	Mol wt 259.8

The methyl stearate melted at 37.5°;  $n_D^{55} = 1.4300$ . The free acid obtained on saponification melted at 71.5–73°.

$C_{18}H_{36}O_2$ (284)	Calculated	C 76.05, H 12.67
	Found	" 76.17, " 12.77
	"	Mol wt 286.6

The residue from the first distillation weighed 0.321 gm. After saponification and crystallization a very small quantity of an acid was obtained, which melted at 67.5–68° and which had a molecular weight of 300.

From the quantities of the ester fractions and their indices of refraction, it was estimated that the reduced acids consisted of about 63 per cent palmitic acid and 37 per cent of stearic acid, the trace of the higher unidentified acid being disregarded.

The iodine number, 84.3, of the liquid fatty acids indicated the absence of acids containing more than one double bond. In a separate experiment 10 gm. of liquid fatty acids were prepared from the lecithin and brominated in petroleum ether solution at –15°. No insoluble bromides separated. It is evident therefore that no highly unsaturated acids were present in the lecithin.

*Solid Saturated Fatty Acids*—The solid fatty acids obtained in the first hydrolysis were insufficient in amount to permit of adequate fractionation and purification. An additional 36 gm. of lecithin was therefore hydrolyzed and the fatty acids were separated into solid and liquid acids. The solid acids, weighing 2.4 gm., were purified by the Twitchell method (7) and converted into the methyl esters. The esters were fractionated as described above. The two purest fractions obtained corresponded in properties to methyl palmitate and methyl stearate and no indication of either lower or higher acids was found. The esters on saponification gave palmitic acid and stearic acid of correct melting point and composition. For the sake of brevity analytical data are not given. From the indices of refraction of the esters, it was estimated that 43 per cent of palmitic acid and 57 per cent of stearic acid were present in the mixture.

#### *Examination of Water-Soluble Constituents*

*Isolation of Barium Glycerophosphate*—The aqueous solution from which the fatty acids had been extracted was freed of sulfuric

acid quantitatively with barium hydroxide, after which the solution was neutralized to phenolphthalein with barium hydroxide. The precipitate which separated was barium phosphate and weighed 0.315 gm., representing 3.2 per cent of the total phosphorus. The filtrate on addition of alcohol gave a precipitate of barium glycerophosphate, which was collected, washed, and dried *in vacuo*. The substance, a white amorphous powder, weighed 3.438 gm., corresponding to 33.2 per cent of the total phosphorus. The low recovery of phosphorus in the above determination was due as found later to incomplete precipitation of the glycerophosphate. Addition of more barium hydroxide to the filtrate gave a copious precipitate of barium glycerophosphate. In a separate hydrolysis, we were able to recover all of the phosphorus of the lecithin in the hydrolysis products—even the fatty acids were found to contain a trace of phosphorus. The barium glycerophosphate was purified by precipitation from aqueous solution with alcohol until a snow-white powder was obtained that was easily and completely soluble in water. For analysis the substance was dried at 105° *in vacuo*.

$C_2H_7O_6P\text{Ba}$ (307.4)	Calculated.	Ba 44.69, P 10.08
	Found.	" 44.49, " 9.69

The barium glycerophosphate showed a slight levorotation,  $[\alpha]_D^{23} = -1.31^\circ$ . By the method of Karrer and Solomon (8), it was found to contain about 18 per cent of  $\beta$ -glycerophosphoric acid.

*Isolation of Choline*—The filtrate from the barium glycerophosphate was acidified with hydrochloric acid, evaporated to dryness *in vacuo*, and the residue was extracted with absolute alcohol. The solution was made up to 100 cc. and aliquots were taken for examination. A nitrogen determination (Kjeldahl) showed that the solution contained 90.43 per cent of the lecithin nitrogen.

From 10 cc. of the solution we obtained 0.85 gm. of choline chloroplatinate or about 92 per cent of the anticipated amount. This figure is in agreement with the observation of MacLean (9) that choline is precipitated in 92 per cent yield by chloroplatinic acid. The choline chloroplatinate was recrystallized from dilute alcohol and for analysis was dried to constant weight at 100° *in vacuo*.

$(C_8H_{14}NOCl)_2PtCl_4$  (616.2). Calculated, Pt 31.68; found, Pt 31.69



*Cephalin Hydrolysis Products*—The methods used in the hydrolysis and in the examination of the cleavage products of cephalin were identical with those described under lecithin, hence the results only will be given in this report. The purified cephalin, 21.77 gm., after hydrolysis gave 13.84 gm. or 63.57 per cent of fatty acids which were separated into 10.33 gm. or 84 per cent of liquid acids and 1.97 gm. or 16 per cent of solid acids.

*Liquid Fatty Acids*—The liquid fatty acids had an iodine number of 77.6. After catalytic hydrogenation no liquid saturated acids were found. The reduced acids were methylated and the methyl esters were fractionated as described above. A trace, 37 mg., of the most volatile portion of the esters melted at 11–15°; the balance was a mixture of methyl palmitate and methyl stearate from which pure fractions were obtained. After saponification of the esters pure palmitic and stearic acids were isolated. As estimated from the indices of refraction of the esters, the mixture contained 42 per cent palmitic acid and 58 per cent stearic acid. No evidence was found for the presence of any higher acid.

*Solid Fatty Acids*—The solid fatty acids melted at 54–55° and the molecular weight as determined by titration was 264.9. The acids were first purified by the Twitchell method (7) and then esterified and the esters were fractionated by distillation. From the indices of refraction, we estimate that the esters consisted of 44 per cent methyl palmitate and 56 per cent methyl stearate and no indication was found of the presence of any other acids. After the pure ester fractions had been saponified pure palmitic acid and stearic acid were isolated and identified by melting point, molecular weight, and analyses.

*Glycerophosphoric Acid*—The aqueous portion of the hydrolysis mixture was treated as described under lecithin. There were obtained 0.83 gm. of barium phosphate and 7.217 gm. of barium glycerophosphate. The purified barium glycerophosphate when tested by the method of Karrer and Solomon (8) gave a slight amount of the double barium salt corresponding to 9 per cent of  $\beta$ -glycerophosphoric acid. In aqueous solution the substance showed no optical activity. For analysis the substance was dried to constant weight at 105° *in vacuo*.

$C_8H_{17}O_6PBa + 1.5H_2O$ (334.4).	Calculated.	Ba 41.08,	P 9.27
	Found.	" 41.08, 41.09,	" 9.17, 9.27

*Aminoethyl Alcohol*—The filtrate from the barium glycerophosphate was evaporated to dryness *in vacuo* and the residue was extracted with absolute alcohol. The solution was concentrated to 15 cc. and mixed with an alcoholic solution of picrolonic acid. The picrolonate, which separated on cooling, was filtered, washed, and dried. The substance, which weighed 5.06 gm., was recrystallized from alcohol and separated in yellow prisms. The picrolonate melted with decomposition at 223–225° and there was no depression of the melting point when mixed with an authentic specimen of aminoethyl alcohol picrolonate.

$C_{12}H_{15}N_5O_6$  (325). Calculated, N 21.53; found, N 21.06

Complete details of the work here reported may be obtained from the thesis by L. F. Salisbury on file in the Yale University Library. The thesis also contains a review of the elementary analyses and hydrolysis products of phospholipids from various sources, as reported in 132 published papers.

It is a pleasure to acknowledge our indebtedness to The Fleischmann Laboratories, New York, and to Dr. C. N. Frey for support given this investigation.

#### SUMMARY

1. The mixed phospholipids of yeast have been separated into lecithin and cephalin. The products were purified until the lecithin was free from amino nitrogen and all of the nitrogen of the cephalin was in the amino form.

2. On hydrolysis both phospholipids gave about 64 per cent of fatty acids.

3. The component fatty acids of both the lecithin and cephalin were found to be very similar, consisting of 84 to 86 per cent of liquid acids. The liquid acids on catalytic reduction gave a mixture of palmitic and stearic acids. The solid acids likewise contained only palmitic and stearic acids

4. The water-soluble portion of the hydrolysis products of lecithin consisted of optically active glycerophosphoric acid and choline.

5. The water-soluble fraction from cephalin contained optically inactive glycerophosphoric acid and aminoethyl alcohol.

6. Hydrolecithin and hydrocephalin were prepared and analyzed.

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## THE BLOOD CHOLESTEROL IN THE CAROTID ARTERY, VENÆ CAVÆ, AND PORTAL VEIN\*

By FREDERICK H. SHILLITO, EMILY H. BIDWELL, AND  
KENNETH B. TURNER

*(From the Department of Medicine, College of Physicians and Surgeons,  
Columbia University, and the Presbyterian Hospital, New York)*

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The literature of cholesterol metabolism abounds in reports of quantitative differences in the blood cholesterol brought about by passage through various organs or tissues. If an increase occurred, the tissue was assumed to have added to the circulating cholesterol either through actual synthesis or by release from storage. A decrease was ascribed to destruction or storage of cholesterol.

Interest has centered primarily on the lungs, which are said to remove cholesterol from the blood stream. Although the claim has not been accepted without controversy, the greater number of papers seems to favor this view. Such changes, if present, would be of obvious importance. We were prompted, therefore, to report our observations in an attempt to elucidate this point.

The subject was opened by Abelous and Soula (1) who found in dogs that the blood serum from the right heart had a higher cholesterol content than that from the left heart. They explained this difference by the assumption that the lungs destroyed cholesterol. Others (2-13) reported similar findings for cholesterol or total fat. This difference was said to disappear in pancreatized animals (7-9, 14) only to be restored by insulin. Hoppe (15), however, found that the total fat of the blood from the right heart was only occasionally higher than in the femoral artery, and Markowitz and Mann (16), also working with total fat, reported no difference in the blood proximal or distal to the lungs. In acute pulmonary edema produced in rabbits by intravenous injections of adrenalin, it was claimed that the blood lipids in the

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left heart were actually higher than in the right (17). Recently Kuriyagawa *et al.* (18) reported the total lipid of the plasma to be greater in the right heart than in the carotid artery following the administration of olive oil to dogs. The cholesterol was relatively the same in blood from the two sites. These authors concluded that the lungs had no specific rôle in fat metabolism.

Increased pulmonary ventilation was said to augment the power of the lungs to lower blood cholesterol, while, conversely, asphyxia decreased this ability (2). Bugnard (3, 19) believed that the change in the cholesterol of the blood on passage through the lungs was more apparent than real, as a rise in acidity caused a shift of cholesterol from cells to plasma in venous blood, while the lungs by lowering the carbon dioxide caused a reverse movement. However, Scheffer (20) denied that the distribution of cholesterol between cells and plasma was influenced by carbon dioxide or oxygen.

In other locations, it has been reported that cholesterol is higher in the splenic, femoral, and renal veins than in the corresponding arteries (4, 11). In the portal vein it was said to be higher than in the subhepatic vein, in the superior vena cava higher than in the inferior (11).

#### EXPERIMENTAL

In our experiments fifteen cats and two dogs were used. The animals were deeply anesthetized by pentobarbital sodium (nembutal) administered intraperitoneally. A tracheotomy was performed, a tracheal catheter inserted, and artificial respiration instituted.

A cannula was placed in the right carotid artery. Next, the sternum was removed without opening the pericardium but giving exposure to the venæ cavæ. Finally, an upper abdominal incision was made, permitting approach to the portal vein.

A sample of arterial blood was obtained from the carotid. Blood was withdrawn from both venæ cavæ, as it was thought that the thoracic duct chyle, mingling with the blood in the superior vena cava, conceivably might cause a difference in cholesterol content. Finally, blood was taken from the portal vein. These four samples of blood were taken as nearly simultaneously as possible. Certainly not more than a minute elapsed between the

time when the first and last specimens were secured. The total cholesterol content of the whole blood, except as noted below, was determined by the method of Bloor, Pelkan, and Allen (21).

*Control Animals*—In the first group were four cats used as controls. Food was withheld from these animals on the day previous to operation except for a small meat ball. Blood samples were obtained as described above. The results are shown in Table I.

In an additional group of three cats similarly prepared, the analyses were made upon the serum instead of upon the whole blood in view of the work of Bugnard (3, 19), who believed that the difference between the cholesterol content of blood in the right

TABLE I  
*Cholesterol of Whole Blood and Serum (Mg. per 100 Cc)*

Cat No	Whole blood				Cat No	Serum			
	Superior vena cava	Inferior vena cava	Carotid artery	Portal vein		Superior vena cava	Inferior vena cava	Carotid artery	Portal vein
1	116	114	116	112	5	114	116	114	113
2	135	134	132	136	6	168	164	166	164
3	110	112	114	114	7	101	102	104	104
4	132	131	129	120	Average	128	127	128	127
Average	123	123	123	121					

and left hearts was due to a shift of lipid from cells to plasma, the value for the whole blood remaining constant. The results are given in Table I.

From Table I it is evident that the whole blood and serum cholesterol values show no significant variation whether the sample is taken before or after passage through the lungs or from the portal vein.

*Cholesterol-Fed Animals*—We next endeavored to ascertain whether a recent, heavy cholesterol feeding had any effect upon the distribution of cholesterol in the blood in these four locations. Accordingly, four cats and two dogs were each given 6 to 12 hours before operation a meat ball containing both lean meat and fat to which cholesterol had been added. The cats received 2 gm. of

cholesterol.<sup>1</sup> The dogs were given 10 gm. Blood samples were secured in the usual way. The results are shown in Table II.

It is apparent that here again the cholesterol values are strikingly constant for an individual animal. Only two discrepancies occur. The rather low value for the portal vein blood in Cat 8 can be explained by a wet syringe. The sample from the inferior vena cava in Cat 9 was partly clotted.

TABLE II  
*Whole Blood Cholesterol (Mg per 100 Cc)*

	Superior vena cava	Inferior vena cava	Carotid artery	Portal vein
Cat 8	162	156	160	149
" 9	135	114	127	125
" 10	116	117	114	116
" 11	166	161	160	161
Dog 303	216	211	211	213
" 304	184	187	189	

TABLE III  
*Whole Blood Cholesterol (Mg. per 100 Cc)*

Cat No	Days of I	Superior vena cava	Inferior vena cava	Carotid artery	Portal vein
12	2	163	166	163	164
13	8	139	138	134	136
14	14	97	96	95	96
15	14	162	156	162	157

*Iodized Animals Fed Cholesterol*—As a final step we sought to determine whether iodization caused any disturbance in the distri-

<sup>1</sup> The feeding of cholesterol in a meat ball may be objected to by some on the ground that the cholesterol might be slowly and very incompletely absorbed without the addition of more fat. We were not interested in this report in the degree of absorption. That some absorption of cholesterol takes place even in the absence of fat has been shown by Versé (22) and by Knudson (23). It should also be pointed out that one of the present authors found increases up to 1000 per cent in the blood cholesterol of rabbits following the oral administration of pure cholesterol mixed with grain, but without the addition of fat (24).

bution of the blood cholesterol from various sites. Accordingly potassium iodide was given to four cats. Each animal received 1 gm. a day for from 2 to 14 days. Cat 14 displayed definite evidence of toxicity on this dosage, and it is of interest that its blood cholesterol values are the lowest for the total series of fifteen cats. The other three animals in the group were apparently not disturbed by the iodide administered.

Cholesterol was given before operation as in the last group. Blood samples were obtained as before. The results are shown in Table III. It is apparent that again no significant variation in the blood cholesterol is present.

#### SUMMARY

1. The level of the blood cholesterol in the carotid artery, superior and inferior venæ cavae, and portal vein is the same for a given animal. This is true whether whole blood or serum is used, and whether or not cholesterol was administered before operation.

2. Specifically, no effect of passage through the lungs upon the blood cholesterol was demonstrable.

3. Potassium iodide given in large doses for from 2 to 14 days did not produce a variation in distribution of blood cholesterol in the vessels from which samples were taken.

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## STUDIES ON MYOGLOBIN

### I. THE SOLUBILITY OF MYOGLOBIN IN CONCENTRATED AMMONIUM SULFATE SOLUTIONS

By VINCENT E. MORGAN

*(From the Department of Physical Chemistry, Harvard Medical School, and the Fatigue Laboratory, Morgan Hall, Harvard University, Boston)*

(Received for publication, October 15, 1935)

The crystallization by Theorell (8) of one of the hemoglobins of muscle, horse heart myoglobin, has made possible the more accurate study of this interesting protein, and opened the way for the investigation of other members of the series. Previously the question of the existence of a protein in the muscle resembling, but nevertheless distinct from, blood hemoglobin was in doubt. Theorell's work seems to have proved quite definitely that there is such a protein. The present report is the first of a projected series of investigations of the myoglobins. That of the horse heart was chosen because it is readily obtained and crystallized as carboxymyoglobin.<sup>1</sup> Moreover, the hemoglobin of the horse has been repeatedly studied (2). This study of the solubility of myoglobin has yielded conclusive evidence—supplementing that of Theorell (9) on molecular weights, oxygen dissociation curves, and absorption spectra—of the distinct nature of these two hemoglobins from the same species.

Investigations of solubility usually are carried out with the object of characterizing the saturating body, perfecting methods for its separation and purification, or determining its activity coefficients and thereby learning something of the physical properties of the molecule upon which these depend. The solubility of horse myoglobin in concentrated phosphate and sulfate solu-

<sup>1</sup> In the preliminary work for this study, attempts were made to prepare the myoglobin from steer and pig hearts by the Theorell procedure. In both instances no crystals were obtained. This indicates a species difference among the myoglobins similar to that present among the blood hemoglobins.

tions characterizes its behavior and facilitates its separation from the iron-containing proteins of the blood which have higher molecular weights and are more readily salted-out

*Preparation*—Preparations 2, 3, and 5 were made from fresh horse hearts obtained through the kindness of the Massachusetts State Antitoxin Laboratory at Forest Hills. The animals were bled to death under chloroform, the heart removed immediately, and the coronary system perfused with about 6 liters of normal saline solution. Theorell's procedure (8) was then followed exactly, except that litmus paper was used as a pH indicator and all the dialyses were carried out through cellophane membranes. The procedure consists essentially of extraction of the finely ground tissue with water, followed by precipitation with lead acetate. The filtrate is then repeatedly dialyzed against solutions of saturated ammonium sulfate. Characteristic fan-shaped clusters of needle crystals appeared after the second dialysis.

Preparations 7 and 8 were made similarly to Preparations 2, 3, and 5. There was, however, one important modification. In the first place an attempt had been made to study the solubility of myoglobin in concentrated phosphate solutions. This failed because myoglobin appears to be extremely soluble in phosphate buffers at pH 6.6, even when the concentration of phosphate is as high as 3 M. As a result of this observation a simple method is available for the almost quantitative separation of hemoglobin and myoglobin. A calculation based on the equation given by Green (2) for the solubility of horse carboxyhemoglobin in phosphate solutions shows that under these conditions only  $9 \times 10^{-4}$  gm. per liter, or 1 part in 1,000,000, of hemoglobin would be left in solution. In view of these facts, Preparations 7 and 8 were made from horse hearts which had not been perfused and which were at least a day old when the preparation began. They had been preserved in the cold from the time of extirpation and were washed thoroughly to remove as much blood as possible before grinding and extraction. Theorell uses phosphate to remove the lead after the lead acetate precipitation in his procedure. Because of the solubility relationships discussed above, after the lead acetate precipitation the solution was brought to approximately pH 6.6 and a phosphate concentration of 3 M by adding the calculated (3) quantities of primary and secondary potassium phosphate in

the solid form. After vigorous stirring the mixture was centrifuged and the clear supernatant liquid treated according to the Theorell procedure from this point on.

In all cases the crystals were purified by washing two or three times with saturated ammonium sulfate solution made from the c.p. salt. They were then dissolved in a minimal amount of water and recrystallized by again dialyzing against a saturated ammonium sulfate solution. The crystals were washed a few times after each crystallization. In Preparations 7 and 8 recrystallization was effected twice and the final washing carried out with a solution of specially purified ammonium sulfate. This was prepared by recrystallizing Baker's c.p. Analyzed salt twice from water solution by the gradual addition of absolute alcohol. The alcohol was removed by careful drying in air on a Buchner funnel and then in the oven at 110°. All ammonium sulfate solutions used were saturated and adjusted colorimetrically to pH 6.6 with ammonium hydroxide.

*Solubility*—The solubility determinations were carried out by the methods previously reported from this laboratory. On account of the small quantity of saturating body available, however, no analyses could be carried out in duplicate. To overcome this difficulty equilibrations were continued for at least 43 hours before filtration and in some cases as long as 73 hours. The scattering of the points (Fig. 1) is in part due to the impracticability of performing duplicate analyses, but the large number of determinations overcomes uncertainty regarding the salting-out range. As many as six different points were obtained from a single myoglobin preparation by adding a slightly more dilute ammonium sulfate solution to the saturating body after the filtration from the previous point had been completed and repeating the equilibration for the next point. Thus the equilibrium was approached always from the side of resolution. The ammonium sulfate used with Preparations 2, 3, and 5 was Baker's c.p. Analyzed salt, the solutions made from it being filtered before use. The twice recrystallized ammonium sulfate described above was used with Preparations 7 and 8. All solutions were adjusted to pH 6.6 with ammonium hydroxide before being added to the saturating body and the whole mixture was saturated with carbon monoxide by bubbling the gas through it for about 15 minutes.

Aliquots of the filtrate were heat-coagulated, washed free from ammonium sulfate by the method of Sorensen (7), and subsequently analyzed for nitrogen. The heat coagulation was carried out by adding to each aliquot 20 cc of a 3.2 M phosphate buffer, pH 6.6, 30 cc. of a saturated solution of recrystallized potassium sulfate, and enough distilled water to bring the volume to about 100 cc. The potassium sulfate served to insure complete precipitation of the myoglobin. The solution was heated on a boiling

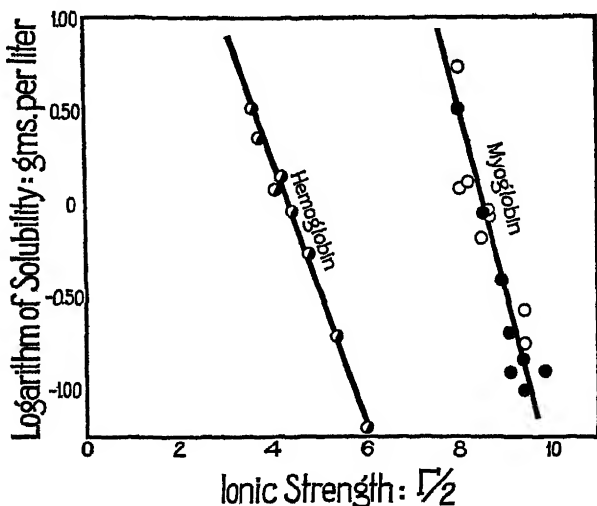


FIG 1 A comparison of the solubilities of the carboxyhemoglobin and carboxymyoglobin of the horse at pH 6.6 and 25° in ammonium sulfate solutions. Carboxyhemoglobin ○ (2); carboxymyoglobin, Preparations 2, 3, and 5 ○, Preparations 7 and 8 ●.

water bath for at least 30 minutes with frequent shaking and then allowed to stand overnight. On the following day it was filtered through Whatman No. 42 paper, repeatedly washed with large volumes of boiling distilled water, and nitrogen analyses performed by the Kjeldahl method on both the residue and on aliquots of the filtrate. The residue nitrogen was calculated as myoglobin on the basis of the nitrogen content (16.65 per cent) determined by Theorell (9) p. 64 and the nitrogen of the filtrate as ammonium sulfate.

The pH determinations were made with the quinhydrone electrode on each filtrate from Preparations 7 and 8 immediately after filtration. The presence of so much ammonium sulfate in the solutions makes necessary a "salt correction," the magnitude of which for 25° we failed to find in the literature. A comparison of the work of Linderstrøm-Lang (6) and the recent publication of Hovorka and Dearing (5) indicates that the correction cannot amount to more than 0.09 to 0.15 pH unit over the range of ammonium sulfate concentrations employed. Even after the maximum corrections have been applied, the resultant pH of every solution in which it was determined lies between 6.25 and 6.75, a range over which the solubility probably changes but little.

Density determinations were made actually only on solutions from Preparations 7 and 8. Since ammonium sulfate is the principal constituent other than water in these solutions, density was calculated as a linear function of ammonium sulfate molality and the result applied in calculating the density of the solutions from Preparations 2, 3, and 5 on the basis of their ammonium sulfate content. The densities were used in calculating  $\mu$  and the solubility per 1000 gm. of water.

The great difference between the solubilities of carboxyhemoglobin and carboxymyoglobin is shown by Fig. 1. The importance of this difference in behavior as a basis for separation has already been pointed out. It is proof of the non-identity of the pigments. The lower molecular weight of myoglobin determined by Theorell ((9) p. 46) is consistent with its greater solubility.

In solutions of high salt concentration, the solubilities of many proteins can be described by the linear formula

$$\log S \text{ (per 1000 gm. H}_2\text{O)} = \beta - Ks\mu$$

or

$$\log S \text{ (per liter)} = \beta' - Ks' (\Gamma/2)$$

where  $S$  is the solubility in gm.,  $\beta$ ,  $\beta'$ ,  $Ks$ , and  $Ks'$  are constants,  $\mu$  is the ionic strength per 1000 gm. of  $\text{H}_2\text{O}$ , and  $\Gamma/2$  is the ionic strength per liter. These constants have been calculated from the data for myoglobin by two methods and the results serve to define the limits of error. The first method consisted in the application to the data obtained from all five

preparations of a formula<sup>2</sup> for the most probable straight line towards which a series of values tends. In the second method, points obtained with Preparations 7 and 8, which fall close to a straight line, were used to calculate the constants by means of simultaneous equations. The points that deviate the most from this straight line (Fig. 1) are those from the earlier and less accurate experiments. In our opinion the best constants are those derived from the last experiments, which were carried out with the most carefully purified material and after the details of technique had been perfected.

TABLE I  
*Constants for Egg Albumin and Hemoglobin and Myoglobin of Horse*

	Per liter solution		Per 1000 gm H <sub>2</sub> O	
	$\beta'$	$Ks'$	$\beta$	$Ks$
Horse carboxyhemoglobin . . . . .	3 09	0 71	2.80	0 57
" carboxymyoglobin* . . . . .	8 00	0 94†	6 12	0 57
Egg albumin . . . . .	6 56	1.11	6 22	0 91

\* These constants are deduced from the measurements on Preparations 7 and 8. The statistical average of all results yields for  $\beta$  and  $\beta'$  5.45 and 7.00, and for  $Ks$  and  $Ks'$  0 51 and 0 83, respectively.

† In a recent review (1) the preliminary work for this paper was referred to and a tentative value of  $Ks$  given. The value given above is more nearly correct, being based on more extensive and more careful experimental work

The constants defining the behavior of horse myoglobin in concentrated salt solution are compared in Table I with those from horse hemoglobin<sup>3</sup> (2) and egg albumin (1). It is interesting to

<sup>2</sup> The formula used for the calculation of the most probable straight line towards which a series of values tends is as follows.  $y = Rx - Rxm + ym$ , where  $R = (N\sum xy - \sum x\sum y)/(N\sum x^2 - (\sum x)^2)$ ,  $N$  = the number of observations,  $xm$  = mean value of  $x$ , and  $ym$  = mean value of  $y$ .

<sup>3</sup> The solubility of human hemoglobin (4) appears to be intermediate between that of horse hemoglobin and myoglobin. In a phosphate solution of ionic strength 6, in which horse carboxymyoglobin is extremely soluble, the solubility of both horse and human carboxyhemoglobin is not more than 0 001 gm. per liter. Although the solubilities of these blood hemoglobins are nearly alike in so concentrated a phosphate buffer, their separation from each other is almost quantitative at an ionic strength of about 4, owing to

note that when the results are calculated on the basis of 1000 gm. of  $H_2O$ ,  $K_s$  values for both hemoglobin and myoglobin are identical, although the values of  $\beta$  are still very different. Egg albumin has been included in Table I because both its molecular weight and volume are very close to those of myoglobin.

The author is greatly indebted to Professor E. J. Cohn for his invaluable interest and advice during the course of this research.

#### SUMMARY

1. The solubility in concentrated solutions of ammonium sulfate of carboxymyoglobin prepared from horse heart has been investigated at pH 6.6 and 25°. It is adequately described by the equation  $\log S = 8.00 - 0.94 (\Gamma/2)$ .

2. Horse heart carboxymyoglobin is quite soluble in buffer solutions at pH 6.6 up to a phosphate concentration of at least 3 M, in which solution the solubility of horse carboxyhemoglobin is less than 1 part in 1,000,000. The great difference in solubility demonstrates the great difference between these proteins, and yields a method for their characterization and quantitative separation.

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the far higher solubility of human than of horse hemoglobin. In this respect the former resembles horse myoglobin more closely than does the hemoglobin of the same species





## INVESTIGATIONS IN ENZYMATIC HISTOCHEMISTRY

### I. DISTRIBUTION OF ARGINASE ACTIVITY IN RABBIT KIDNEY

By LEOPOLD WEIL AND J. OWEN ELY

*(From The Biochemical Research Foundation of the Franklin Institute,  
Philadelphia)*

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The fundamental investigations of Linderstrøm-Lang and Holter in enzymatic histochemistry have provided means for investigating the specific enzymatic metabolism of the cell. Cellular enzymes are not uniformly distributed throughout a given organ but are connected with a definite morphological structural element of the tissue depending on the physiological function. The work of Linderstrøm-Lang, Holter, and Sjøeborg-Ohlsen (1, 2) and the recent work by Glick and Biskind (3) indicate this relationship.

The present work correlates the arginase activity of rabbit kidney with a definite morphological structural element of this organ. Much attention has been given to this enzyme, particularly within the last few years. The mechanism of arginase activity and its participation in tumor metabolism, for example, have been widely investigated by Edlbacher and coworkers (4, 5), Waldschmidt-Leitz, McDonald, and coworkers (6), Klein and Ziese (7), Purr and Weil (8), and Weil (9). The importance of this enzyme in urea formation has been emphasized by Krebs and Henseleit (10).

In our histoenzymatic investigation the arginase activity was measured by the method developed by Linderstrøm-Lang, Weil, and Holter (11). Our results show that arginase activity is present in the cortex only of the rabbit kidney and runs parallel with the number of proximal convoluted tubule cells. No connection was found between the arginase activity and any other type of cell. The medullary portion of the kidney showed no arginase activity. The possibility that the medulla may contain some substances which inhibit the activity of this enzyme was excluded, since the addition of inactive medullary tissue to cortical tissue did not

inhibit the arginase activity of the latter. It is, therefore, evident that the arginase of the rabbit kidney is specifically connected with the cells of the proximal convoluted tubules.

Whether the enzyme is secreted by the cells of the proximal convoluted tubules or is transported to them from some other source cannot be answered at present. Neither do we desire to express an opinion as to the physiological function of this enzyme, until the systematic investigations of other kidney enzymes, which we have undertaken, expand our knowledge of the enzymatic metabolism of the kidney.

#### EXPERIMENTAL

In correlating the arginase activity of kidney tissue with the histological structure it is necessary to select a species of animal possessing a kidney of suitable size. If the kidney is too small, like that of the rat, one encounters difficulty in the histological portion of the work, while with a kidney too large in size difficulties are encountered in the freezing-microtome technique. Rabbit kidney was found to be of convenient size. Male animals were used in all of the experiments.

Immediately after killing the animals, by bleeding, the kidney was removed and placed in a refrigerator ( $-5^{\circ}$ ) until the tissue was somewhat firm but not well frozen. This usually required from 1 to 2 hours. A longer time in the refrigerator is not advisable, since considerable damage to the cells was observed after 2 hours. By use of a cork borer 2.5 mm. in diameter in a drill press a column of tissue was cut from the kidney immediately after removal from the refrigerator. The length of this column was usually too long for the microtome technique, so it was cut into halves and each portion studied separately. Each half of the column of tissue was in turn placed on a rotary freezing-microtome table, a piece of muscle tissue being used as a base. With a cold microtome knife the necessary slices of tissue for the enzyme studies were cut. All efforts to use the same tissue column for the histological portion of the work as was used for the enzymatic investigation failed, because the severe freezing during the microtome technique injured the cell structure of the kidney too greatly, although it had no effect on the arginase activity. For this reason we adopted the procedure of Linderstrøm-Lang, Holter, and Sjøeborg-Ohlsen (2)

and used for our histological work the hollow cylinder of tissue (about 9 mm. in diameter) immediately surrounding the hole from which the column had been removed for the enzyme estimation. Preliminary studies had shown that the histological structure of the column used for the enzyme work was practically identical with that of the outside cylinder of tissue. This identity of the two portions is confirmed later by the relatively small calculated error. After removal of the column of tissue for the enzymatic study the outside cylinder was fixed in Zenker's solution, dehydrated, and embedded in paraffin by the customary procedure.

### *Histological Description*

The adult mammalian kidney is composed of a capsule, the renal tubules, lymph vessels, and blood vessels. The renal tubules, originating with the renal corpuscles (glomeruli) and ending in the ureter, are composed of the following successive parts: renal corpuscle (glomerulus) with its capsule of Bowman, the proximal convoluted tubule, descending and ascending limbs of Henle's loops, distal convoluted tubule, and the collecting tubule, or excretory duct. The histological portion of this study is the problem of distinguishing these types of tubules from one another, and making estimations of the relative occurrence of each type at known locations in the tissue.

The embedded tissue was cut into sections  $6\ \mu$  in thickness, mounted on a slide, and stained with hematoxylin and eosin for the microscopic examination.

### *Microscopic Procedure*

For the estimation of the amounts of the various types of tubules and for making cellular counts, the stained sections were placed in a microprojector which was in the vertical position so that the image was projected onto a paper placed on the supporting table. With a suitable magnification the various kinds of tubules may be recognized and cellular counts made. Four low power microscopic fields (ocular  $25\times$ , objective  $2, 6\times$ , Leitz) of the same diameter as the hole in the paraffin-embedded cylinder of tissue were selected around the hole in the sections. In these fields the area occupied by glomeruli and blood vessels (blood vessels largely in the medulla) and lymph spaces were outlined on the projected image and

their areas measured by means of a planimeter. In each of the low power fields mentioned above four oil immersion fields, making a total of sixteen fields in each section, (ocular 8 X, objective HI 90 (1.25), Leitz) were selected for cellular counts (nuclear) of the various types of tubules. In the cortex and medulla, cellular counts made in a particular paraffin section showed a relatively low variation. This variation, however, was greater in the boundary zone between the cortex and the medulla but still sufficiently small that a uniform curve of distribution could be obtained, as indicated by the calculated errors.

A more detailed description of the method used by us in making the cellular counts and the calculations of experimental errors is given in the more extensive work of Linderstrøm-Lang, Holter, and Sjøborg-Ohlsen (2).

Shrinkage of the tissue by fixation and embedding must be taken into consideration in order to enable one properly to compare the curve of the enzyme distribution with that of the cellular distribution curves. By dividing the length of the frozen column used for enzyme work by the length of the outside paraffin-embedded cylinder of tissue used for histological purposes a factor is obtained which enables one to fit the two curves together. This shrinkage factor with rabbit kidney by the described procedure was usually about 1.17, and was used as described by Linderstrøm-Lang, Holter, and Sjøborg-Ohlsen (2).

*Method of Determining Arginase Activity*—The method of Linderstrøm-Lang, Weil, and Holter (11) with one slight modification was used for the determination of arginase activity. The use of a buffer was found to be necessary in order to maintain the same conditions in both the blank and the main determination. When no buffer was used, the pH of the enzyme in the blank was about 7.0, while that of the main enzyme determination was 9.5. Since self-digestion of the tissue was found to be dependent on the pH of the mixture, being great at pH 7 and practically nil at pH 9.5, a buffer was introduced. For each determination 3.5 c.mm. of 0.1 M glycine-NaOH buffer at pH 9.5 were used.

*Dependence of Arginase Activity upon Thickness of Microtome Slice of Tissue*—In order to investigate the possible dependence of arginase activity upon the thickness of the microtome slice of tissue, rabbit kidney was removed from the animal immediately after death by bleeding, and prepared for the microtome technique

as previously described. The column of tissue was cut through the kidney from side to side by use of a cork borer 25 mm. in diameter. Such a column of tissue contained cortex on each end with medulla in the middle portion. At certain points in the column of tissue a 25  $\mu$  section and, immediately adjoining, two 12.5  $\mu$  sections were taken and the arginase activity determined in its initial and its fully activated condition. In the case of the initial activity, the microtome slice of tissue was placed in a micro-reaction vessel into which 7 c.mm. of distilled water had been measured. After the addition of 3.5 c.mm. of 0.1 M glycine-NaOH buffer at pH 9.5 and 7 c.mm. of 0.1 M arginine substrate at pH 9.5 the activity was measured by the usual method (11). When measuring the full activity of the enzyme, cysteine and ferrous sulfate were used as an activator system (9). The cysteine solution used was prepared by taking 20 mg. of cysteine hydrochloride, neutralizing at pH 7, and diluting to a volume of 25 cc. The ferrous sulfate solution was prepared by diluting 0.4 cc. of 0.1 N ferrous sulfate solution to a total volume of 25 cc. From each of these two solutions 3.5 c.mm. were taken for activation of the enzyme. The use of a greater concentration of the activator caused no increased activation of the enzyme. In measuring the full activity of the enzyme the microtome slice of tissue was placed in the reaction vessel containing 3.5 c.mm. of the ferrous sulfate solution previously described. After addition of 3.5 c.mm. of the previously described cysteine solution the reaction mixture was allowed to stand for 1 hour in order to obtain the maximum activation. After this period of activation 3.5 c.mm. of 0.1 M glycine-NaOH buffer at pH 9.5 and 7 c.mm. of 0.1 M arginine substrate at pH 9.5 were added. The measurements were all made by means of a micropipette (12). The blank determinations were carried out in the same manner as the main determination except that the arginine substrate<sup>1</sup> was placed as a drop on the wall of the microreaction vessel. The incubation period was 2

<sup>1</sup> It was found that arginine used as a substrate should contain 20 volumes per cent of glycerol. With only an aqueous solution of arginine, it was observed that, especially with the blank determination, the arginine crystallized out of the drop on the wall of the reaction vessel. The addition of the acetone-alcohol mixture used in titrating the ornithine formed (11) failed to dissolve the crystals of arginine, thus producing an error. The use of 20 volumes per cent of glycerol in the arginine solution avoids entirely the crystal formation.

hours at 37°. Arginase activity in Fig. 1 is expressed in c.mm. of 0.05 N alkali. Fig. 1 shows that practically no difference in the arginase activity is obtained by the use of one slice of tissue 25  $\mu$  in thickness or two slices 12.5  $\mu$  in thickness, proving that contact between enzyme and substrate was complete.

*Influence of Length of Extraction Period upon Arginase Activity of Microtome Slice of Kidney Tissue*—In order to study the influence of the length of the extraction period on the arginase activity, rabbit kidney tissue was prepared and the enzyme determinations were carried out as previously described. At certain

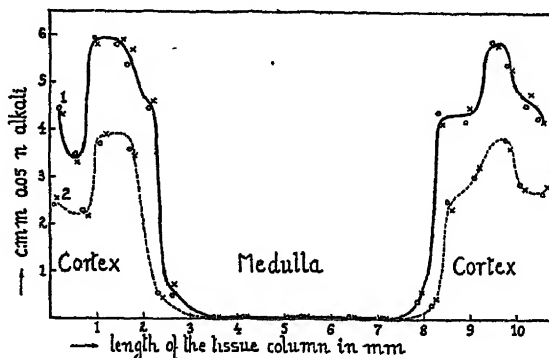


FIG. 1. The dependence of arginase activity upon the thickness of the microtome slice of tissue. On Curve 1 the circle indicates the full activity of one 25  $\mu$  section, the cross full activity of two 12.5  $\mu$  sections; on Curve 2 the circle indicates the initial activity of one 25  $\mu$  section, the cross initial activity of two 12.5  $\mu$  sections.

points in the column of tissue, two adjoining sections 15  $\mu$  in thickness were taken. For initial activity, one of these sections was allowed to stand in 7 c.mm. of distilled water for 1 hour and the other section for 4 hours. For measuring the full activity one section was placed in 3.5 c.mm. of ferrous sulfate solution and allowed to stand for 1 hour. The other section treated in the same manner was allowed to stand for 4 hours. After this period of extraction, the arginase activity was measured as previously described, the mixture being incubated for 3 hours at 37°, and expressed in c.mm. of 0.05 N alkali. Fig. 2 shows that the prolonged extraction time had no effect on the arginase activity. The

arginase was found to be dissolved only slightly and was apparently connected with the tissue structure proper. Diffusion must, therefore, have been complete; this seems very probable, since the substrate has a low molecular weight. Similar observations were made by Linderstrøm-Lang and Holter (1) on dipeptidase and by Weil (13) on trypsin.

*Microunits for Arginase Activity*—It was necessary to establish a microunit for arginase activity in order to enable us to make comparisons between arginase activity and cellular distribution. For this reason it was necessary to establish a relation between

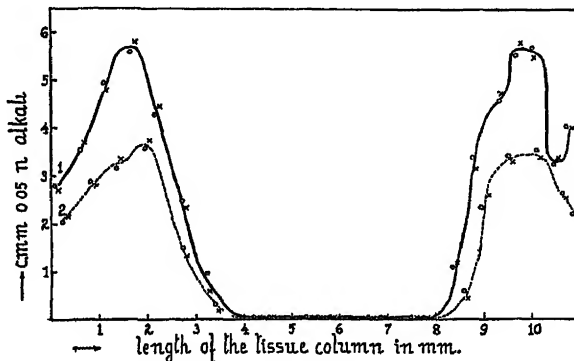


FIG 2 Influence of the length of the extraction time on the arginase activity of slices of kidney. On Curve 1 the circle indicates the full activity after 1 hour's extraction time, the cross full activity after 4 hours extraction time; on Curve 2 the circle indicates the initial activity after 1 hour's extraction time, the cross the initial activity after 4 hours extraction time.

enzyme quantity and degree of splitting of the substrate. Since in all of our enzyme activity determinations, an incubation period of 3 hours at 37° was used, the same conditions were selected in establishing this relationship. Rabbit kidney was minced and suspended in 5 parts of a 20 per cent glycerol-water solution. After 24 hours extraction in this solution (bacterial infection was avoided by adding a few drops of toluene), the suspension was filtered. Increasing amounts of the kidney extract were used by taking 2, 4, 6, 8, 10, 14, 16, 18, 20, and 24 cc. of the extract and diluting to a volume of 25 cc. in each case. The final glycerol concentration in each determination was 7 per cent. For each



determination 7 c.mm. of prepared enzyme solution were taken. After the addition of 3.5 c.mm. of 0.1 M glycine-NaOH buffer at pH 9.5 and 7 c.mm. of 0.1 M arginine substrate at pH 9.5 the reaction mixture was incubated for 3 hours at 37° and the ornithine formed estimated as previously described (Table I).

The microarginase unit selected was that quantity of enzyme which under the experimental conditions was able to produce in 3 hours at 37° an equivalent of 1 c.mm. of 0.05 N alkali, which corresponds to the decomposition of  $8.75 \times 10^{-4}$  mg. of arginine into ornithine and urea (Fig. 3).

TABLE I  
*Relation between Quantity of Enzyme and Degree of Arginine Splitting*

Kidney extract (1:5) in 7 c.mm.	Arginase activity, c.mm. 0.05 N alkali
c.mm.	
0.56	1.72
1.20	2.96
1.68	3.98
2.24	4.86
2.80	5.50
3.36	6.14
3.92	6.70
4.48	7.15
5.04	7.56
5.60	8.20
6.72	8.70

*Enzyme and Cellular Distribution in Cortex and Medulla of Rabbit Kidney*—In order to ascertain the real connection between the arginase activity and the various types of cells, it was necessary to compare the curve of enzyme activity at various points in the column of tissue through the kidney with the curves for the various types of cells at corresponding points in the kidney tissue. As described in the histological portion of this paper, the cells of glomeruli, proximal convoluted tubules, descending and ascending limbs of Henle's loop, distal convoluted tubules, and the collecting tubules must be taken into consideration. Fig. 4 shows the arginase activity distribution and cellular counts in the cortex (largely glomeruli, proximal convoluted tubules, and distal convoluted tubules). Fig. 5 shows the arginase activity distribution curve

and cellular counts in the medulla (largely collecting tubule cells, and ascending and descending Henle's loop tubule cells). The enzyme activity is expressed in Figs. 4 and 5 in units for one slice of tissue containing 73  $m\lambda$  investigated enzymatically.<sup>2</sup> The

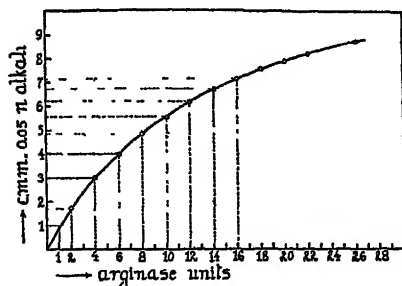


FIG. 3. Arginase units

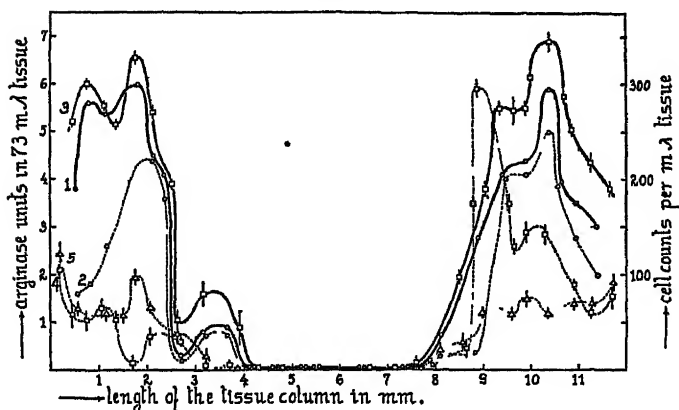


FIG. 4. Enzyme and cellular distribution in the cortex of rabbit kidney. Curve 1 shows the full activity of arginase, Curve 2 the initial activity of arginase, Curve 3 cellular counts of proximal convoluted tubules, Curve 4 cellular counts of distal convoluted tubules, Curve 5 per cent area of glomeruli tubules.

cellular counts were made as previously described, and calculated for  $m\lambda$  ( $1 m\lambda = 10^{-3} \times \text{c.m.m.}$ ) of tissue. For each cellular count

<sup>2</sup> The data for the glomeruli in the cortex of the rabbit kidney are expressed in Fig 4 in per cent area, because of the difficulty of making cellular counts in these structures. An arbitrary unit was used in making the curve.

made the calculated error (2) is indicated by a vertical line across the curve. Fig. 4 shows that a parallelism exists between the enzyme activity curve and the cellular counts for the proximal convoluted tubules only. The possibility that the arginase activity in the medulla is inhibited will be excluded by experiments to be described later. For the measurement of the arginase activity a 15  $\mu$  section of 2.5 mm. in diameter was used. After a 3 hour incubation period at 37° the initial and full activity was measured as previously described. Fig. 4 shows the relation between arginase activity and cells of the proximal convoluted tubules, glomeruli, and distal convoluted tubules. A parallelism exists between the enzyme activity and the cells of the proximal convoluted tubules only. Fig. 5 shows that no relation exists between arginase activity and the different types of tubule cells in the medulla.

Figs. 6 and 7 show in two additional cases that the rabbit kidney arginase activity parallels the cellular count of the proximal convoluted tubules. The cellular counts were made as previously described. The arginase activity is indicated in units per 73  $m\lambda$  of tissue.

*Influence of Medulla on Arginase Present in Cortex*—Figs. 1, 2, and 4 to 7 indicate that the medulla shows no arginase activity and that the arginase activity is dependent on the cells of the proximal convoluted tubules present in the cortex. The possibility that the medulla might contain an inhibitor for arginase occurred to us. However, as Table II shows, the addition of medullary substance did not inhibit the arginase activity exhibited by cortical tissue. These results were obtained with sections 15  $\mu$  in thickness from a column of tissue 2.5 mm. in diameter. From a certain portion of the tissue column two adjoining sections (which may be considered identical histologically) were taken from the cortex. The arginase activity of one of these sections was measured alone, while with the other section the same determination was made after the addition of a similar section from the medulla. Three such experiments on tissue from different portions of the cortex were made. The results indicate the full activity of the enzyme in each case, the determinations being carried out as previously described and expressed in c.mm. of 0.05 N alkali. The results shown in Table II indicate that the medulla has no inhibiting action on the

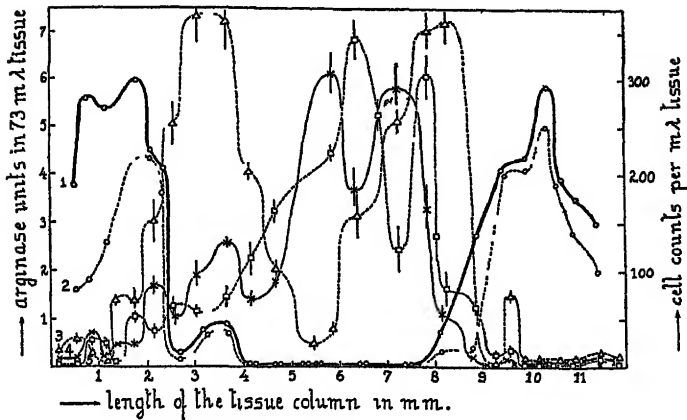


FIG. 5. Enzyme and cellular distribution in the medulla of rabbit kidney. Curve 1 shows the full activity of arginase, Curve 2 the initial activity of arginase, Curve 3 cellular counts of ascending Henle's loop tubules, Curve 4 cellular counts of descending Henle's loop tubules, Curve 5 cellular counts of collecting tubules

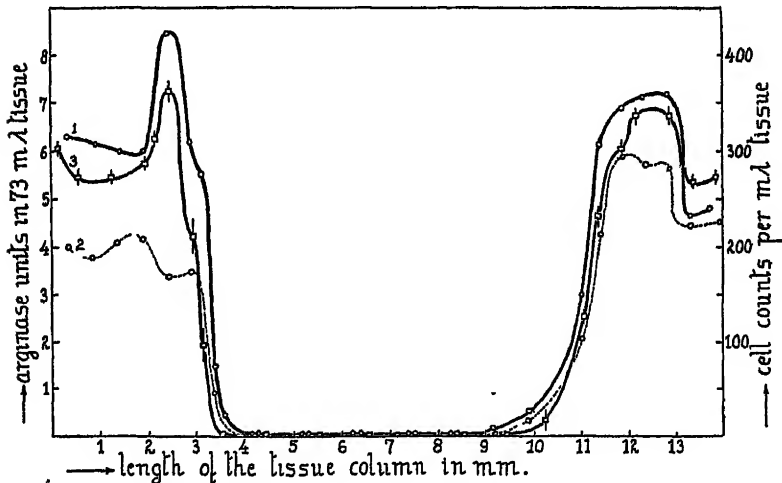


FIG. 6. Enzyme and proximal convoluted tubule cell distribution in rabbit kidney. Curve 1 shows the full activity of arginase, Curve 2 initial activity of arginase, Curve 3 cellular counts of proximal convoluted tubules.

arginase activity of the cortex and shows no arginase activity itself. In order to be certain that the desamidase of the kidney (14) has no effect upon the ornithine formed, experiments with ornithine and kidney tissue were carried out at pH 9.5. No increase or decrease in the titration value for the ornithine was found.

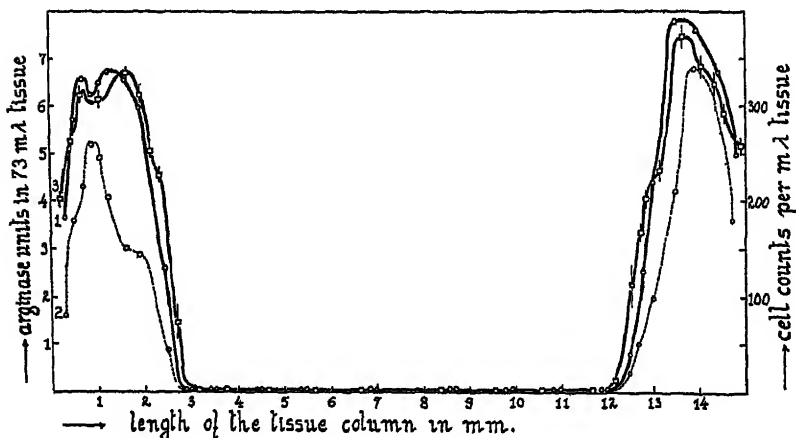


FIG. 7 Enzyme and proximal convoluted tubule cell distribution in rabbit kidney. Curve 1 shows the full activity of arginase, Curve 2 initial activity of arginase, Curve 3 cellular counts of proximal convoluted tubules.

TABLE II

*Influence of Medulla on Arginase Present in Cortex*

The results are expressed in c mm. of 0.05 N alkali.

Experiment No	Arginase activity shown by 15 $\mu$ section		
	Cortex	Medulla	Cortex-medulla
1	4.86	0.0	4.92
2	5.22	0.0	5.16
3	5.30	0.0	5.26

SUMMARY

It has been shown that the microarginase method, as used in these experiments, is suitable for histoenzymatic studies.

The arginase activity of the rabbit kidney is specifically connected with the cells of the proximal convoluted tubules.

No correlation was found between the arginase activity and cells of the other structural elements of the rabbit kidney.

The medulla of the rabbit kidney is entirely free of arginase activity and does not contain any inhibitor of this enzyme.

*Addendum*—While this paper was in press, Klein and Ziese (15) reported that manganous sulfate activates arginase. In view of this, the medulla portion of the kidney, found inactive by our activation method, was re-investigated with manganous sulfate as activator. These experiments, however, showed no activation over a period of 24 hours, thereby confirming the previously found inactivity of the medulla of the kidney.

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# THE ENZYMIC SYNTHESIS FROM THYROID DIODOTYROSINE PEPTONE OF AN ARTIFICIAL PROTEIN WHICH RELIEVES MYXEDEMA\*

BY WILLIAM T. SALTER AND OLOF H. PEARSON

*(From the Collis P. Huntington Memorial Hospital of Harvard University, Boston)*

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This report describes the enzymic synthesis of an artificial protein which relieves clinical myxedema. The enzyme used was pepsin, applied under conditions suggested by Wasteney and Borsook (1, 2) which reverse its usual digestive effect. As previously reported, the starting material or substrate was the diiodotyrosine peptone obtained by the peptic digestion of human thyroglobulin (3, 4). This material has hitherto been considered to be calorigenically inert. Coincident with peptone linkage, and associated with the formation of larger molecules, the material has acquired marked physiological potency. It is the purpose of this communication to describe the preparation and properties of the resultant colloidal, iodine-containing substance.

Observations by Salter, Lerman, and Means (5) had indicated that the total organic iodine, rather than the thyroxine content of thyroid substance, was the better index of its ability to relieve myxedema. These facts led to the paradoxical conclusion that the so called "inactive"<sup>1</sup> iodine fraction of thyroglobulin was in reality calorigenically potent so long as it existed as an integral part of the thyroglobulin molecule. The "inactive" diiodotyrosine fraction has constituted two-thirds to four-fifths of the total

\* This work was made possible by grants from the International Cancer Research Foundation and the Ella Sachs Plotz Foundation.

<sup>1</sup> The term "inactive iodine-containing fraction of thyroglobulin" indicates the iodine-containing moiety of thyroglobulin which cannot be isolated as thyroxine. This "inactive" fraction is soluble in dilute acid (in contrast to thyroxine and its derivatives), and yields crystalline diiodotyrosine after careful intensive hydrolysis.



organic iodine found in human material studied in Boston.<sup>2</sup> It was important, therefore, to learn whether this fraction could become active under appropriate conditions.

Harington and Salter (6) found that after isoelectric precipitation of such a digest to remove the thyroxine fraction the iodine-containing peptones which remained in solution contained no thyroxine. This "acid-soluble" peptone, studied by Harington and Randall (7), has already been shown by Lerman and Salter (8) to be without effect in myxedema in a standard daily dosage containing 0.5 mg. of iodine. Would the effectiveness reappear provided the relatively inert peptone were recombined into an artificial thyroglobulin of colloidal dimensions?

The work of Wasteney and Borsook (1, 2) suggested a possible procedure. These investigators obtained from Witte's peptone and from peptonized egg albumin artificial colloids which they named "plasteins." The theoretical rationale of their procedure was peptic synthesis of peptones, according to the law of mass action, by reversing the usual digestion reaction. The thermodynamic considerations governing such a reversed process have been treated at length by these authors. For the moment we are concerned not with their interpretation but with the observed phenomenon. In the present investigation, treatment with pepsin has yielded an iodine-containing, non-dialyzable fraction which relieves myxedema in human patients.

#### EXPERIMENTAL

*Chemical Procedure*—Two types of surgically excised human goiter were used as sources of human diiodotyrosine peptone: the primary hyperplastic gland of Graves' disease and the multiple colloid adenomatous goiter often diagnosed as "toxic adenoma."<sup>2</sup> Both types of material were treated similarly. After mincing of the tissue, the thyroglobulin was twice extracted with 0.02 N NaOH, and repeatedly precipitated isoelectrically as described elsewhere (6). The thyroglobulin was incubated for 72 hours at 37° and pH 1.5 with 0.2 per cent Merck's pepsin, then heated to 100° in 20 minutes, brought to pH 5.0, and the precipitate filtered off after standing 8 hours.

<sup>2</sup> For this material we are greatly indebted to Dr. Tracy B. Mallory of the Massachusetts General Hospital and to Dr. Shields Warren of the Deaconess Hospital, Boston.

In a typical preparation, starting with thyroglobulin from glands of patients suffering from exophthalmic goiter, after digestion, coagulation, and isoelectric precipitation, 3900 cc. of diiodo-tyrosine filtrate were removed, containing 109 mg. of iodine. This was concentrated under reduced pressure at 60–70° to a brown syrup, containing 105 mg. of iodine in a volume of 250 cc.

For the synthesis of the plastein, 150 cc. of the acid-soluble peptone (at pH 5.0) were used, containing 63 mg. of iodine. To this amount were added 4 cc. of concentrated hydrochloric acid (with rapid shaking) to make the pH 3.9. Then, 30 cc. of a 10 per cent aqueous solution of Merck's granular pepsin were added, and stirred into the solution, which remained clear. The liquid was

TABLE I  
*Experiments with Pepsin and Ultrafiltered Peptone*

The total volume in each experiment = 6 cc. of peptone solution containing 320 mg of nitrogen and 2.0 mg of iodine.

Enzyme preparation	Concentration of enzyme	Total enzyme used	Total N of synthetic product	Total I of synthetic product	N I of product	Weight of product*	Product formed per mg enzyme
	mg per cc	mg	mg	mg		mg	mg.
Merck's u s r. pepsin	16.7	100.0	31.2	0.47	61	231	2
Northrop's crystalline pepsin	0.33	2.0	24.0	0.30	80	178	89
	0.13	0.8	26.4	0.33	80	195	244
	0.08	0.5	22.2	0.35	63	164	328
	0.03	0.2	14.2	0.20	71	105	525

\* Estimated from nitrogen

then heated to 60° in a water bath. Within a few minutes it became turbid. After 45 minutes at 60° there was a considerable precipitate present.

At this temperature, over the course of the next 30 minutes, 16 cc. of approximately 2.5 N NaOH were added to change the pH to 5.5. 100 cc. of additional water were then added, and the rapidly flocculating material was at once centrifuged down at 2000 R.P.M. for 20 minutes. After the supernatant liquor had been decanted, the precipitate was shaken up with 200 cc. of water and again centrifuged down. Two further such washings were given. The washed precipitate was finally suspended in water to a volume of 210 cc. It contained 18 mg. of iodine, i.e. 29 per cent of the

original iodine. A few drops of toluene were added to the bottle before stoppering, and the material was kept iced for several weeks before being tested for biological potency

*Rôle of Pepsin*—Experiments were conducted to exclude the possibility that the colloidal pepsin used contributed materially to the product. As seen in Table I, the highest yield was obtained with an excess of pepsin. Nevertheless, by using crystalline pepsin, kindly donated by Dr. John H. Northrop, it was possible to show that very small concentrations of enzyme exhibited significant effectiveness. The ratio of enzyme to product (in terms of mass) could be reduced below the analytical error.

*Clinical Assay*—Clinical tests were made upon untreated myxedema patients according to the method described by Salter, Lerman, and Means (5). The daily dose of material was equivalent to 0.5 mg. of iodine. Both the original ineffective diiodotyrosine concentrate (before treatment with pepsin) and the artificial protein resulting from treatment of this (identical) concentrate were tested. The results are described elsewhere (4). In brief, the inactive iodine-containing substance from thyroid had become active in standard dosage during the synthesis of the plastein.

*Properties of Artificial Protein Made from Diiodotyrosine Peptone Concentrate*

*General Properties*—The artificial protein shows several properties characteristic of the behavior of proteins. As judged by minimal solubility, it shows a sharp isoelectric point around pH 5.5. It gives the biuret reaction. It is precipitated by trichloroacetic acid in a concentration of 2 per cent. It is coagulable by heat; and the coagulum, although very insoluble in dilute hydrochloric acid alone, is readily digested on the addition of pepsin at pH 1.5. The artificial protein denatures very readily in 0.1 M NaCl solution or even on standing at the isoelectric point with a trace of salt present. When freshly prepared, it is definitely soluble in urea solutions, but not in solutions of sodium chloride, in which it apparently undergoes denaturation at once. It immediately dissolves when sodium hydroxide is added to the isoelectric suspension to a concentration of 0.004 N. If excess of electrolyte be avoided, the alkaline solution can be quickly changed (with dilute hydrochloric acid) to an acid solution without precipitation.

Addition to the acid solution of an equal volume of saturated aqueous sodium chloride salts-out the artificial protein, in accordance with the phenomenon described by Osborne and Campbell (9).

*Dialysis*—In order to test qualitatively the relative molecular size of the protein preparation with respect to that of the peptone, each was dialyzed in cellophane tubes (No. 300, plain) against a 20-fold volume of liquid which was kept agitated with bubbling air.<sup>3</sup>

The initial iodine concentration in all cases was 2 mg. of iodine per cent. The protein was dialyzed against 0.004 N sodium hydroxide, which kept the material dissolved. The peptone concentrate, at pH 5.0, was dialyzed against distilled water. Dialysis was continued for 4 to 13 days at 4°. The outer liquid was renewed daily in the case of the peptone.

The (outer) dialysate from the peptone gave a strong biuret reaction after 3 hours, and this positive reaction continued for some 5 days, despite daily renewal of the dialyzing fluid. The protein, on the other hand, never showed a clearly positive biuret reaction in the dialysate after the 1st day. After 4 to 13 days, the dialysis was interrupted, and the contents of the membrane were removed and analyzed for iodine and for nitrogen.

The results are summarized in Table II. They show that most of the artificial protein failed to penetrate the cellophane, whereas most of the peptone did pass through the membrane. These data offer good ground for supposing that the mean size of the protein molecule is definitely greater than that of the original peptone.

*Composition*—The nitrogen and iodine contents of several preparations are presented in Table III. The iodine and nitrogen were determined in aliquots of a suspension of material, and these figures were referred to the nitrogen content of artificial protein dried for 1 hour in a hot air oven at 100°. The data indicate that the artificial proteins varied in composition, and suggest that the nature of the peptone concentrate from which each was made was an important determining factor in the nature of the product. It is interesting that the composition is not very unlike that of natural thyroglobulin.

<sup>3</sup> These tests were carried out in the Department of Physical Chemistry, Harvard Medical School, with the technical help of Mrs. Ethel S. Newman.

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It is particularly striking, however, that in the process of peptic synthesis a greater content of iodine might result (with reference to nitrogen) in the artificial protein than was found in the original peptone. This point is illustrated in Table III, which shows a considerable difference in the respective ratios of iodine to nitrogen in some instances. Preparation II-a was exceptional in this respect.

A further striking fact is presented in the analyses of the starting materials and of the products of peptic synthesis for their respective thyroxine contents. This is also illustrated by Table III.

TABLE II

*Dialysis of Artificial Protein and of Peptone from Which It Was Made*

The initial concentration of iodine = 2 mg per cent Peptone dialyzed against water; artificial protein against 0.004 N NaOH.

Preparation No	Material dialyzed	Duration of dialysis	Result of dialysis*	
			Total N not diffusible	Total I not diffusible
		<i>days</i>	<i>per cent</i>	<i>per cent</i>
I. MCAG†	Peptone	5	15	
	Artificial protein	4	88	82
II-a "	Peptone	8	17	15
		11	22	14
	Artificial protein	8	67	82
III. EXG‡	Peptone	13	10	10
	Artificial protein	10	69	63
VI. "	Peptone	11	17	17
	Artificial protein	11	80	85

\* Cellophane (plain No 300) membrane used.

† Initial thyroglobulin obtained from multiple colloid adenomatous goiter.

‡ Initial thyroglobulin obtained from exophthalmic goiter.

As was to be expected from the relative ineffectiveness of the di-iodotyrosine peptone in treating myxedema, the thyroxine content of the peptone was essentially nil within the experimental error of the analytical procedure. It was rather surprising, however, to find that the plastein, as judged by the best analytical procedures now available (10, 11), contained considerable moiety of "thyroxine iodine." Furthermore, after alkaline hydrolysis, this thyroxine-like, acid-soluble fraction relieved myxedema. The "thyroxine moiety" was about the size of that found in the natural

TABLE III  
Composition of Peptone and of Artificial Protein

Preparation No.	Material analyzed	N in dried material*	N I (in terms of mass)	Calculated I in dried material	Relative concentration of I referred to N	Apparent thyroxine content	Yield in terms of I
		per cent		per cent		per cent total I	per cent
I. MCAG†	Peptone	14.3	113	0.11			
	Artificial protein	12.8	45.7	0.30	2.5	52 (Harington and Randall)	32
II-a "	Peptone	13.2	127	0.09		36 (Blau)	
	Artificial protein	11.8	79.3	0.15	1.6	4 (Harington and Randall) 7 (Blau) 38 (Harington and Randall)†	16
III. EXG§	Peptone	15.2	142	0.08		3 (Harington and Randall)	
	Artificial protein	11.8	51.7	0.23	2.8	30 (Harington and Randall)†	29
	Dialyzed artificial protein	12.7	52.8	0.24	2.7		
IV. " Crystalline pepsin	Peptone	14.5	137 157	0.11 0.09		5 (Harington and Randall)†	
	Artificial protein	13.5	63.1	0.21	2.2 2.5		17
V MCAG† Crystalline pepsin	Peptone	13.4	126	0.11		1 (Harington and Randall)†	
	Artificial protein	12.9	74.3	0.17	1.7	13 (Harington and Randall)†	15

\* Corrected for ash

† Initial thyroglobulin obtained from multiple colloid adenomatous goiter.

‡ 8 hours hydrolysis in boiling 2 N NaOH.

§ Initial thyroglobulin obtained from exophthalmic goiter.

|| After filtering through the cellophane membrane.

TABLE III—*Concluded*

Preparation No	Material analyzed	N in dried material* per cent	N I (in terms of mass)	Calculated I in dried material per cent	Relative concentration of I referred to N	Apparent thyroxine content per cent total I	Yield in terms of I per cent
VI. EXG§ Crystalline pepsin	Peptone	13.6	95.7	0.14		0 (Harington and Randall)† 6 (Blau)	17
	Artificial protein	12.7	56.6	0.22	1.7	29 (Harington and Randall)† 24 (Blau)	
	Dialyzed artificial protein	12.7	49.8	0.25	1.9		

human thyroglobulin used in these experiments. Experiments now in progress to isolate thyroxine have not as yet yielded crystalline material, although the general behavior of this fraction resembles crude thyroxine in process of isolation. The effect of alkaline hydrolysis reported by Abelin (12) is of interest in this respect.

*Yield*—As appears in Table III, the efficiency of plastein synthesis, in terms of the iodine recovered, varied in different preparations. This variation appeared to be due in part to variation in the composition of different peptone concentrates, because duplicate preparations from the same concentrate gave identical yields, however different these might be from results with other concentrates. The yield also depended upon the concentration of enzyme used. In general, the recovery of one-third of the iodine was the maximal yield to be expected.

After recovering approximately 30 per cent of the total peptone iodine in the form of artificial protein, an attempt was made to increase the total recovery by repeating the peptic synthesis upon the residue of diiodotyrosine peptone. To this end, the unchanged peptone was saved after removal of the new formed protein. The solution was then concentrated until its iodine composition corre-

sponded to the original concentration. Peptic synthesis was then repeated. The result was disappointing. To be sure, some (additional) new protein was formed, but it contained only about 5 per cent of the iodine taken.

#### DISCUSSION

It is the purpose of this paper merely to describe the production of an active hormone resembling natural thyroglobulin from the large moiety of thyroid iodine which has hitherto been discarded as inert (6) in the purification of thyroxine. The clinical assays on myxedema patients, reported elsewhere (4), leave no doubt that the artificial protein is calorigenically potent in standard dosage.<sup>4</sup> This activity, therefore, lends additional weight to the view advanced by Means, Lerman, and Salter (14) that the thyroxine iodine, alone, as determined by present methods, is not the best index of the calorigenic activity of thyroid products, because the active artificial protein, here described, arises from the non-thyroxine fraction of thyroglobulin.

It is not yet clear how ingested thyroid acts effectively with all its diiodotyrosine fraction in myxedema, whereas diiodotyrosine uncombined with protein is relatively useless when fed alone. The relationship between the peptones of thyroxine and of diiodotyrosine must be studied further to explain the high activity of their combination (15). In this connection we propose to consider the possibility that proteases in tissues may synthesize hormones from the end-products of gastrointestinal proteolysis. Meanwhile, in view of the idea currently held that *only* the thyroxine iodine in thyroglobulin can be potent (16), it seemed important to present evidence to the contrary. The constitution of the artificial protein is being studied further in this laboratory and will be the subject of a subsequent communication (17). For clarity's sake, however, the authors' interpretation of the facts now at hand is summarized here.

The authors accept the hypothesis of Harington and Barger (18) that duodotyrosine is the natural starting material for the syn-

<sup>4</sup> The authors are particularly impressed by the pharmacological variations imposed by species differences (13), and wish to emphasize the fact that the present work is based upon human responses to material of human origin



thesis of thyroxine by the gland. The authors believe that the present communication indicates that the acid-soluble moiety of thyroid iodine is in fact convertible into an active hormone. Evidence will be presented elsewhere (19) that an activation of this moiety may occur *in vivo*. Experiments now in progress point to a naturally occurring protease in the thyroid cells as a possible means by which this might be accomplished. Proteases are known, indeed, to exist in many tissues (20), and Hedin (21) has actually studied the synthetic activity of a protease extracted from the spleen. Indeed, Scott's (22) unsuccessful attempt to renew the activity of digested insulin was based on similar reasoning.

A cogent question is the actual chemical constitution of the newly activated organic iodine. Two intensive hydrolyses of the artificial protein have failed to yield crystalline thyroxine. Nevertheless, the partition of iodine in the successive chemical steps of the procedure follows closely that described by Harington ten years ago for the isolation of thyroxine from natural thyroglobulin. One fraction of the acid-insoluble material so obtained contained approximately 10 per cent of iodine and 6 per cent of nitrogen. By contrast, the hydrolysis of the initial acid-soluble peptone has yielded a negligible amount of organic iodine insoluble in dilute aqueous acid. In short, the technical complexity of the isolation procedure prevents, at present, any conclusion further than that the physical properties of the organic iodine change after peptic synthesis. This fact, together with the biologic potency of the acid-insoluble fraction, is interpreted as meaning progress in the enzymic synthesis of thyroxine from diiodotyrosine. Whether the authors have actually produced thyroxine or merely a closely related intermediate substance is as yet not clear.

#### SUMMARY

Thyroglobulin from human thyroids was subjected to peptic digestion and, after removal of thyroxine, a solution of diiodotyrosine peptone was obtained which was calorimetrically inert in standard dosage. After concentrating the peptone, it was subjected to a peptic synthesis which reversed the original digestion process. The artificial protein so prepared was an iodine-containing substance of large molecular size, with chemical properties

somewhat resembling the natural thyroid protein. It was found clinically to relieve myxedema as effectively as thyroglobulin in equivalent iodine dosage. As judged by modern analytical methods, it contained an appreciable iodine fraction resembling thyroxine. These results suggest the following conclusions.

The "inactive" diiodotyrosine-containing fraction of peptone produced by pepsin digestion of thyroglobulin is a potential source of active hormone. From this peptone an artificial protein can be obtained by protease synthesis ("ese" action) which reverses the better known proteolytic phenomenon (digestion). The artificial protein resembles natural thyroglobulin in its chemical and biological properties.

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## LIPID COMPOSITION AND PHYSIOLOGICAL ACTIVITY IN THE OVARIES OF PREGNANT GUINEA PIGS

By ELDON M. BOYD

(From the Department of Obstetrics and Gynecology, The University of  
Rochester School of Medicine and Dentistry, Rochester, New York)

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In a previous communication (1) the lipid content of the ovary has been shown to vary with the duration of pregnancy in rabbits. In the present investigation the ovary of the guinea pig has been selected for a similar study. It is generally agreed that double oophorectomy performed on gravid rabbits results in abortion but does not cause abortion if done after the middle of pregnancy or even before in pregnant guinea pigs (2-7). In other words, the ovary or corpus luteum evidently assumes a rôle essential to the maintenance of pregnancy in rabbits, and this has been previously correlated with changes in lipid composition (1). If lipid composition is related to physiological activity, then one would expect to find no significant changes in the lipid content of the ovary during pregnancy in guinea pigs, in which the ovary apparently plays a passive part during most of the condition. This has been herein investigated by following the content of phospholipid and free cholesterol, the two lipids which have been found in previous work to increase in value with increase in physiological activity.

### *Method*

The sexual season of guinea pigs (*Cavia porcellus*) appears to be ill defined, especially in the domesticated variety. According to Marshall (8), the female becomes pregnant more readily in the summer than in the winter. It has been noted in this laboratory that matings were more successful at two periods of the year, one in the spring from April to July, and one in the autumn from September to January. The animals were kept the year round under conditions as nearly constant as possible. A group of about thirty young, virgin, female guinea pigs served for the investigation.

The females were isolated and examined vaginally each day to ascertain the onset of estrus. The guinea pig is a polyestrous animal having a diestrous cycle about every 16 days. During proestrus the vagina becomes filled with mucus and debris from degenerative desquamation of the uterine and vaginal mucosa (8). Coincident with the onset of estrus, the vagina opens and remains so for a period of about 3 days. Ovulation occurs at this time; the female is receptive to the male and capable of being fertilized. When estrus was found to have set in, the doe was isolated and mated with a healthy buck for 3 days. 2 weeks later vaginal examinations were made and if estrus reappeared the mating was considered unfruitful and the animal returned to the original colony. By the 20th day it was usually possible to determine by palpation the presence of a fetus.

The duration of pregnancy was calculated from the middle of the 3 days during which the animals had been mated. Pregnancy in this species lasts about 62 days and the young are relatively well developed at birth; they are able to feed themselves and are not dependent upon the mother's milk. The duration varies somewhat with the rate of recurrences.

A double oophorectomy was performed at various stages in the different animals. In order to obtain values before the 20th day, the abdomen was opened in a group of eleven animals which had been mated 15 days previously. Of these only one was found to be pregnant; this gives some idea of the incidence of successful matings. Ovaries from the remaining ten served as non-pregnant controls.

The ovaries of the gravid animals were similar in appearance to those of non-pregnant guinea pigs. No corpora lutea were visible. The corpora lutea are imbedded in the stroma of the ovary (7). Occasionally cysts were seen but these were as often in the non-pregnant as in the pregnant animals. There was no consistent change in the weight of the ovaries. At all stages the ovaries appeared as small, round or oval, solid, relatively tough, grayish white bodies. They did not macerate when ground with sand as readily as did the ovaries of rabbits (1).

After removal, the ovaries were stripped of adnexal tissue, weighed, cut into fine pieces, and ground with sand in a small mortar. The lipids were extracted with alcohol-ether and the

extracts analyzed by the author's modification of Bloor's oxidative micromethods in the same manner as were the ovaries of rabbits (1).

### Results

The results have been plotted collectively in Fig. 1. Values in the left-hand section of this figure represent the controls and have been spaced so as to give some idea of the variations encountered.

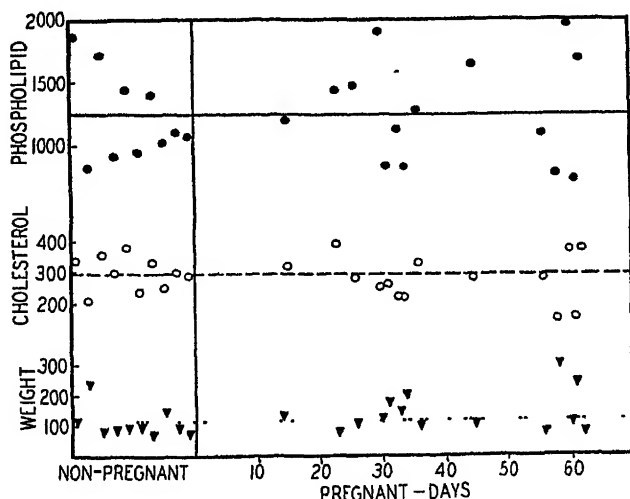


FIG. 1 The phospholipid (upper solid line), free cholesterol (middle interrupted line), and weight (lower dotted line) of ovaries during pregnancy in guinea pigs. The ordinates for phospholipid and free cholesterol are in terms of mg per 100 gm of ovary; the ordinate for weight is in mg.

In these the phospholipid content varied between 818 and 1870 mg. per cent, with a mean of about 1250 mg. per cent. The average value for free cholesterol was about 300 mg per cent, the range extending from 211 to 364 mg. per cent. Most of the ovaries weighed in pairs about 100 mg.; the lowest value was 77 mg. and one exceptionally high value, 250 mg., was encountered. This latter pair of ovaries had the lowest phospholipid and free cholesterol content.

During pregnancy the lipid content of the ovaries varied some-

what from one animal to another but no trends either up or down could be distinguished. Thus, at mid-pregnancy phospholipid varied between 810 and 1902 mg per cent but the mean was about the same as in the controls. Towards term the variation was somewhat greater, between 705 and 1995 mg. per cent, but again there was no change in the average. Values in between varied similarly. On the whole, it is obvious that a straight line at the level of the average of the controls fits best the several points. It may be concluded that no significant change occurred in the phospholipid content and that the ovary during pregnancy contained much the same percentage as that of the controls. It will be recalled that the controls were from animals which had been in estrus 2 weeks previously. Hence the controls were from animals at about the proestrous period, when the corpus luteum probably has become quiescent and a new corpus has not yet formed. The ovary during pregnancy therefore possessed the same amount of phospholipid as the ovary during the proestrous stage.

The results with free cholesterol were of a similar nature. Toward the mid-point of gestation values between 210 and 398 mg. per cent were obtained, with an average around 300 mg. per cent which was the mean of the controls. Near term the percentage ranged from 140 to 385 mg. per cent and again there was no change in the average. Hence a straight line, parallel to that of phospholipid, was best adapted to the various points as plotted in Fig. 1. It may be concluded that free cholesterol does not change in value during pregnancy in guinea pig ovaries, the value being the same as that at proestrus.

The changes in ovarian lipids herein found were thus in marked contrast to those recorded in rabbits (1) in which phospholipid and free cholesterol rose in the first half and fell in the second half of gestation. Since the ovary plays a passive part during the greater part of pregnancy in guinea pigs, an increase in phospholipid and free cholesterol would not be expected if these lipids are related to physiological activity. This was precisely the result herein obtained, which may be taken as additional evidence in favor of this general hypothesis.

#### SUMMARY

Ovaries were removed from twenty-four guinea pigs at various stages during gestation and analyzed for their phospholipid and

free cholesterol content by oxidative micromethods. The phospholipid content of non-pregnant controls at the proestrous stage averaged 1250 mg. per cent. There was no significant variation during pregnancy, the average being the same as in the controls.

Similarly at proestrus the ovary contained a mean of about 300 mg. per cent of free cholesterol. Again there was no significant variation during pregnancy and the mean values were of the same order as those at proestrus. The results were correlated with the fact that guinea pigs may be castrated in the latter half or two-thirds of pregnancy without abortion ensuing and taken to indicate that no increase in physiological activity occurs in the ovaries of gravid guinea pigs.

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## THE BIOLOGICAL ACTIVITY OF THEELOL

BY ROLAND K. MEYER, LLOYD C. MILLER, AND GEORGE F. CARTLAND

(From the Research Laboratories, The Upjohn Company, Kalamazoo)

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In 1930 Marrian (1) reported the isolation and the chemical and biological activity of an estrogenic substance obtained from human pregnancy urine. He found this substance had chemical and biological characteristics differing from theelin which had been isolated and characterized by Veler, Thayer, and Doisy (2) and Butenandt (3). Marrian called this new substance trihydroxyestrin.<sup>1</sup> Later Doisy and Thayer (4) and Butenandt and Hildebrandt (5) also isolated theelol from human pregnancy urine, and the close agreement between the physical and chemical properties of the theelol preparations is presumptive evidence that those isolated in the various laboratories were identical. This statement is also applicable to the crystalline substance isolated from the ether-soluble fraction of human placenta by Collip and his coworkers (6).

However, as one reviews the work which has been published concerning the biological activity of theelol, it is apparent that there is great discrepancy among the reports of the various workers. Thus Curtis and Doisy (7) state that the biological activity of theelol is one-half that of theelin, *i.e.* 1,500,000 rat units per mg. Marrian and Cohen (8) also report an activity of the same order. However, Butenandt and Stormer (9) have found the biological activity of theelol to be approximately one-hundredth that of theelin. Butenandt and Browne (10) assayed a preparation of theelol (Doisy) and reported an activity of about 50 mouse units per mg., which agreed with a parallel assay on their own preparation which they call *Follikel Hormonhydrat*.

<sup>1</sup> Doisy designates the same substance as theelol and Butenandt as *Follikel Hormonhydrat*

In assaying the urine which we were using as the source of theelol we found that it contained relatively small amounts of estrogenic activity. Therefore, we did not expect to obtain a good yield of theelol from this urine, since Doisy and Thayer (4) have stated that approximately 80 per cent of the estrogenic activity of human pregnancy urine can be accounted for as theelol on the basis of yields of crystalline material. However, we were surprised to find that we obtained relatively good yields of a crystalline substance having the chemical and physical properties of theelol but very much less active biologically than theelin, and also less active than reported by Doisy for theelol. A specific example is afforded by the following experiment. A butanol extract from 110 gallons of human pregnancy urine assayed biologically as containing 125,000 rat units. From this butanol extract we were able to isolate a theelin fraction which contained approximately 75 per cent of the original biological activity, or 94,000 rat units. In addition, we obtained a total of 682 mg. of crystalline theelol which, when we assayed it, was not equivalent to more than a total of 50,000 rat units. This crystalline theelol was obtained as three fractions; the first two fractions, which comprised 90 per cent of the total, melted at 274–275° and 271–273° (uncorrected).  $[\alpha]_{5461}^{25} = +75.2^\circ$  in ethanol. The third fraction was not as pure and melted at 265–268° (uncorrected).

Thus we satisfactorily accounted for the biological activity of the original butanol extract. However, on the basis of the report of Doisy and Thayer (4) and Marrian (1) we should have had the equivalent of 1,200,000 rat units in the original butanol extract on the basis that 682 mg of crystalline theelol should have possessed an activity of approximately 1,120,000 rat units, as contrasted with a found biological potency of 50,000 rat units.

These facts, together with the importance of knowing whether the biological activity of theelol is one-half or one-hundredth that of theelin, made it of interest to investigate further the activity of theelol.

#### *Materials and Methods*

Late pregnancy urine was acidified to Congo red and then extracted at room temperature with butanol. The theelol was then

prepared from these butanol extracts by the method of Doisy and Thayer (4) and that of Butenandt and Hildebrandt (11). These methods both yielded similar crystalline compounds; in Table I we have recorded the physical, chemical, and biological characteristics of three lots of theelol prepared by the two methods.

2 gm. of Theelol 76-LCM-2 (m.p. 275-276° uncorrected;  $[\alpha]_{5461}^{25} = +71^\circ$  in ethanol), which had been recrystallized three

TABLE I

*Biological Activity of Theelol and International Standard Theelin*

Preparation	Method of preparation	No. of times recrystallized	M p (uncorrected)	$[\alpha]_{5461}^{25}$	Bioassay		
					Mature spayed rat		Immature rat aqueous*
					Oil	Aqueous*	
			°C	degrees	rat units per mg	rat units per mg	rat units per mg
Theelol 131-GFC-15	Doisy and Thayer	3	276-277	+70	11 1	5 5	
Theelol 76-LCM-3	Butenandt and Hildebrandt	3	275-276	+71	10	5	4000
Theelol 128-GFC-15	" "	10	275-276	+72		5	
Theelin, international standard			252-253		909	1250	1000

\* Aqueous 10 per cent alcohol containing 0.5 per cent sodium carbonate.

times, were recrystallized seven additional times, with the following solvents used in the order named: ethanol, acetone, ethyl acetate, ethanol, methanol, ethanol, ethanol. This procedure yielded 115 mg. of Theelol 128-GFC-15, having the properties described in Table I.

The biological activity of theelol was determined by the vaginal smear method based on that described by Kahnt and Doisy (12). This method makes use of the castrated adult rat which has been primed with an estrogenic substance 1 week prior to its use. The

unit is determined on the basis of the production of a full squamous or predominantly squamous and epithelial vaginal smear in 75 per cent of animals. The injections are made in three equal amounts in 8 hours. We have taken as our unit the amount of hormone required to produce this type of smear in 50 per cent of the animals. We have not classified as negative those animals having smears which contain a very few leucocytes in addition to the squamous and epithelial elements.

In addition to the above method we have used that described by Curtis and Doisy (7). In this method the intact immature female rat is employed and a unit is determined on the basis of the amount of hormone required to produce canalization of the vagina in 60 per cent of the animals within a period of 10 days of the first injection. The hormone is injected twice daily for 3 days. Rats 18 or 19 days old were used by Curtis and Doisy, but in our study we have used animals 19 to 21 days of age.

In both of the methods used in our investigation the estrogenic substances assayed have been administered both in corn oil and in aqueous 10 per cent alcohol containing 0.5 per cent sodium carbonate.

We have experienced considerable variation in the same group of animals as regards their sensitivity to theelin and theelol. This is in agreement with the experience of Curtis, MacCorquodale, Thayer, and Doisy (13). For this reason we have made all but preliminary assays of theelol in parallel with the international standard theelin and have adopted the policy of making repeated assays with ten or more rats at intervals, in contrast to making only one or two determinations on a group of twenty rats. The biological activity given in Table I is the average of several separate determinations. Especial effort has been made to keep comparable all groups of rats used in parallel assays. By using the international standard theelin we have a standard of reference which is available to other laboratories. Use of such a standard in the assay of unknown substances makes it possible and advantageous to report the biological activity of the unknown in terms of the standard.

### *Results*

In Table I are shown the results of the determination of the biological activity of three preparations of theelol as compared

with that of the international standard theelin. These results demonstrate that theelol is relatively an inactive estrogenic substance, when injected in the adult spayed female rat. Our data also show that international standard theelin is approximately 90 times more active biologically than theelol when administered in oil and 250 times when given in 10 per cent alcohol containing 0.5 per cent sodium carbonate. In contrast, the biological activity of theelol when determined by the method in which the immature female rat is used is very great and is approximately 4 times greater than international standard theelin in this regard. Our results obtained with adult spayed rats are at great variance with those reported by Curtis and Doisy (7),<sup>2</sup> Marrian (1), and Marrian and Cohen (8). These workers report the activity of theelol to be one-half that of theelin. The results we have obtained with immature rats are of the same order as those reported by Curtis and Doisy. As has been stated, Butenandt and Störmer (9) have found the biological activity of theelol to be one-hundredth that of theelin when determined by the vaginal smear method, and our results obtained with oil solutions are in accord with their findings.

The recrystallization of Theelol 76-LCM-3<sup>3</sup> seven additional times, which yielded Theelol 128-GFC-15 (Table I) did not result in any change in the physical characteristics or biological activity.

The medium in which the hormone is administered is of importance in determining the number of units in a given amount of theelol. Thus an oil solution injected three times in 8 hours gave results which indicate that the number of rat units is about 10 per mg., as compared to 5 per mg. when theelol is administered in aqueous 10 per cent alcohol containing 0.5 per cent sodium carbonate. If theelol is injected into adult spayed rats as a suspension in aqueous 10 per cent alcohol, the physiological effect is greatly enhanced. Thus, if the concentration of theelol is 0.166 mg. per cc., the biological activity is about 50 rat units per mg. However, if the concentration of theelol is decreased to the

<sup>2</sup> Browne (14) states that Doisy has reported that 0.006 mg. of theelol is required for a rat unit when a full squamous vaginal smear is required in 75 per cent of the animals.

<sup>3</sup> This preparation is the one from which a sample of theelol was converted to theelin by use of potassium bisulfate at high temperature (Cartland, Meyer, Miller, and Rutz (15)).

extent that it is in solution, the activity is of about the same order as when administered in water or in aqueous 10 per cent alcohol containing 0.5 per cent sodium carbonate. These results indicate that the ease with which theelol is absorbed determines to a large extent its biological activity. Thus aqueous solutions of theelol are absorbed more rapidly and are biologically less efficacious than oily solutions or aqueous suspensions from which the rate of absorption is slower.

The activity of theelin is so great as compared with theelol that it is more difficult to determine whether or not suspensions of it would give a greater physiological effect. However, in our hands administration of theelin in an oily solution (injection three times in 8 hours) has given results which indicate that the biological activity is perhaps less when it is administered in oil than in aqueous solution or aqueous 10 per cent alcohol containing 0.5 per cent sodium carbonate.

#### DISCUSSION

The cause of the great difference in the biological activity of theelol as reported by us and that found by other investigators is not definitely known. However, the difference may depend on whether or not a partial or full estrus smear is taken after a positive response. Thayer and MacCorquodale (16) reported that the bioassay of theelol showed an enormous difference, depending upon whether a partial or full estrus smear was considered in the determination of the biological unit. Butenandt and Stormer (9) have treated theelol preparations, having relatively great biological activity, with ketone reagents and thus showed that theelol was contaminated with theelin. They have suggested that the high activity of theelol reported by some investigators is a result of its being contaminated with theelin. However, MacCorquodale, Thayer, and Doisy (17) have failed by use of the same methods employed by Butenandt and Stormer (9) to reveal the presence of theelin in their routine preparations of theelol. Butenandt and Stormer (9) have also suggested that there may be an active and an inactive form of theelol, thus explaining in another way the discrepancy in the reports on the biological activity of theelol. If there are two forms of theelol, we have not obtained any evidence of the second and more active form.

During the process of preparing theelol in our laboratory we have consistently noted that recrystallizations of the first crude theelol result in changes in melting point and specific rotation which indicate increase in purity. Concomitant with these recrystallizations we have observed decrease in biological activity. However, continued recrystallizations do not give indications of any further increase in purity and the biological activity remains constant. This small activity we believe represents a true activity of theelol itself and not that of a more highly active contaminant. This statement is difficult to prove definitely but the small amount of theelol necessary to produce canalization of the vagina of the immature rat in contrast to that possessed by theelin is evidence which tends to support the contention that theelol does have a biological activity—little as regards the adult spayed rat, great when determined in the immature rat.

Browne (14) and Butenandt and Browne (10) have also stated that they believe that theelol has a low grade of biological activity as determined by the vaginal smear method and that this activity is a result of the conversion of theelol to theelin by the tissues. Browne (14) has obtained evidence that less theelol is required to produce estrus in the intact immature rat than in the spayed rat. This, together with other data, has caused him to suggest that the ovary is particularly efficient in the conversion of theelol into theelin.

We have data which show that the amount of theelol, in terms of rat units, necessary to produce uterine bleeding in immature monkeys is much less than theelin (unpublished data). These data, together with certain other facts, indicate that the quantitative relationship between theelol and theelin differs with different methods of assay.

#### CONCLUSIONS

1. Theelol possesses relatively little biological activity when compared with international standard theelin and determined by the vaginal smear method. International standard theelin is approximately 90 times more active than theelol when administered in oil and 250 times when injected in aqueous 10 per cent alcohol containing 0.5 per cent sodium carbonate

2. Theelol is 4 times as active as international standard theelin when determined by the opening of the vagina of the immature rat.



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## THE ESTIMATION OF THE TOTAL LIPIDS AND THE LIPID PARTITION IN FECES\*

By HERBERT C TIDWELL AND L EMMETT HOLT, JR.

*(From the Harriet Lane Home, the Johns Hopkins Hospital, and the Department of Pediatrics, the Johns Hopkins University, Baltimore)*

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For the estimation of total lipids in the feces a number of excellent methods are available, which depend upon primary saponification of the material. When the fat partition is to be determined, however, it becomes necessary to use extraction procedures, a number of which are described in the literature. A method widely used is that of Holt, Courtney, and Fales (1), which is essentially an adaptation of the Roesse-Gottlieb method for the analysis of milk powders. Although subsequent workers (2-5) have altered the procedure in one way or another with a view to improving its accuracy, it remains troublesome and time-consuming. It is the purpose of this publication to present a simplification of this procedure which avoids some of its difficulties, and also to call attention to an error made by previous workers; almost without exception those who have worked with human material have failed to appreciate that the "neutral fat" fraction invariably contains a large proportion of unsaponifiable material; hence the deductions made in regard to fat splitting under various conditions have frequently been unsound. In order to obtain a true picture of fat splitting it is necessary to determine the unsaponifiable matter of the stool fat as well.

The technical difficulties in the procedure of Holt, Courtney, and Fales and its modifications are (1) the tendency for emulsions to be formed during extraction, which often separate out with extreme slowness, and (2) the fact that the determination of "neutral fat," fatty acid, and soap is not made on a single sample,

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but requires several samples and several time-consuming weighings. We have eliminated the difficulty with emulsions by substituting for the Rohrig tube or extraction apparatus a stoppered 50 cc. centrifuge tube, making it possible to break up any emulsions immediately by centrifugation. We have also found it practicable to carry out the estimation of the true neutral fat, unsaponifiable, fatty acid, and soap fractions on a single sample. The lipids other than soap are first extracted with a fat solvent, weighed, and then titrated with alkali to determine the free fatty acid and (by difference) the so called "neutral fat" (neutral fat plus unsaponifiable). The extract is then saponified with alkali, the unsaponifiable material being extracted and weighed, enabling the calculation of all fractions except the soap. The residue from the first extraction, which contains the soap, is acidified to convert the soap into fatty acid which is extracted, weighed, and calculated as soap.

### *Method*

As applied to dried stools, the method is as follows: A sample of powdered stool (1.5 to 2.0 gm., less if quite fatty) is weighed directly into the extraction tube, which consists of a round bottomed 50 cc. centrifuge tube with a narrow mouth, fitted with a cork stopper. The tube is conveniently weighed by standing it in a small beaker on the balance pan. To the weighed stool sample are added 5 cc. of 50 per cent alcohol and the tube is heated on a steam bath, being rotated the while to insure a uniform mixture. The mixture is heated until it begins to boil; it is then cooled and extracted with equal parts of ethyl and petroleum ether (b p. 30–60°). The tube is filled to within an inch of the top, stoppered tightly, and shaken vigorously for a minute after it is seen that no excessive pressure has developed inside the tube. The stopper is then cautiously removed, rinsed with ether, reinserted, and the tube is centrifuged until the supernatant ether is perfectly clear. The extract is blown off as completely as possible, without disturbing the sediment, by means of a wash bottle arrangement with the lower end of the exit tube directed upward so as not to disturb the sediment, into a weighed 250 cc. modified Florence flask<sup>1</sup> containing some very small pieces of porous plate as boiling

<sup>1</sup> The neck of an ordinary 250 cc. Florence flask is shortened to a length of about 1 inch, and a length of about 4 cm. of a round bottomed 50 cc. cen-

stones. It is advisable to pass the ether through a small funnel and filter paper in case sediment is accidentally blown over. The extraction procedure (addition of mixed ethers, shaking, centrifuging, and blowing off of supernatant fluid) is repeated three times (four extractions in all), and the funnel and paper washed with ether, all washings being collected in the modified Florence flask. The solvent is evaporated off, followed by 30 minutes on a steam bath and a similar time in a vacuum desiccator attached to an aspirator. A second heating on a steam bath and second vacuum desiccation are then carried out to remove the last traces of the extracting fluid, after which the flask is weighed. After weighing, 50 cc. of approximately 99 per cent alcohol are added and the flask is warmed to dissolve the fat. The free fatty acids are then titrated while warm with aqueous 0.1 *N* sodium hydroxide while being agitated, phenolphthalein being used as an indicator.<sup>2</sup> The number of cc. of 0.1 *N* base required multiplied by 0.0268 gives the weight of free fatty acids in gm. (calculated from Harrison's (2) figure, 268, for the average molecular weight of fecal fatty acids). The neutral fat (plus unsaponifiable fraction) is the

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trifuge tube is sealed into the bottom of the flask. The purpose of this modification is to provide a flask with volume large enough to collect four washings, and at the same time have a diameter small enough near the base to minimize the error caused by incomplete blowing off of the supernatant fluid

<sup>2</sup> It will be noted that we have abandoned the procedure of Folin and Wentworth (6), used by Holt, Courtney, and Fales, of titrating the fatty acids dissolved in hot benzene with sodium ethylate in favor of solution in hot 99 per cent ethyl alcohol and titrating with aqueous alkali. A series of determinations of stool fatty acids by this method gave the same values as obtained with their recommended procedure. We have checked our procedure by titrations of pure stearic acid and Bureau of Standards benzoic acid, and have found the error to be less than 1 per cent, even when the solution is colored to approximate that of the average stool determination. Under certain circumstances, a shade more accuracy than is obtained by our procedure or by that of Folin and Wentworth can be secured by dissolving the fat in butyl alcohol and titration with sodium butyl alcoholate (an alkali made by treating butyl alcohol with sodium). The latter procedure was suggested by work of Pardee, Hasche, and Reid (7). We find that this has the advantage of giving a darker end-point which is desirable for very dark colored stool fats. Ethyl alcohol, however, has the advantage of being volatile enough to be easily evaporated off after the titration of fatty acids.

difference between this figure and the total weight of the fat in the flask.

The contents of the Florence flask, which, aside from the solvent, now contains neutral fat, unsaponifiable matter, and soaps derived from the titration of free fatty acids are now used to determine the unsaponifiable fraction. Saturated aqueous KOH is then added (0.6 cc. for each gm. of the total extracted *fat* present) and the flask is placed on a steam bath for saponification. The steam is so regulated that 35 to 40 minutes are required to evaporate off all the alcohol. If too much of the alcohol remains unevaporated, difficulties in extraction are encountered. All the material save the unsaponifiable is now in the form of soap and only this latter part (*e.g.* the unsaponifiable) can be extracted by fat solvents. About 5 cc. of water are now added and the material extracted four times with ethyl ether, the ether extract being blown over each time into a 50 cc. extraction tube containing 10 cc. of 5 per cent aqueous KOH solution. After being shaken to wash out any traces of soap that may have been carried over, the extraction tube is centrifuged and the ether extract blown off through a filter into a weighed 250 cc. flask. The four ether extracts are collected in this flask, the ether evaporated off, and the residue which consists of the unsaponifiable matter of the sample is weighed. From this weight the per cent of unsaponifiable matter in the stool and in the stool fat can be calculated, and by difference, the percentage of true neutral fat in the stool can be obtained.

The soap is determined from the stool residue left after the first extraction. Concentrated HCl (2 cc.) is added to the sample along with a few cc. of water. It is warmed and cooled, then extracted four times with mixed ethers, exactly as has been described above. The extract, containing fatty acids derived from soap, is evaporated down and weighed, the final weight being multiplied by the factor 1.1 to convert fatty acid into soap. (This approximate correction was omitted by Holt, Courtney, and Fales.) The total fat is obtained by adding the figures for neutral fat (plus unsaponifiable), free fatty acid, and soap.

A sample calculation follows.

	gm.	gm.
Weight of dried stool (A)	1.837	
" " neutral fat, unsaponifiable, and free fatty acids (B)	0.104	
Titration of (B) required 2.33 cc. of 0.1 N alkali		
Equivalent weight of free fatty acids (C) = $2.33 \times 0.0268$	0.062	
Weight of neutral fat + unsaponifiable (D) = (B - C)	0.042	
" " unsaponifiable (E)	0.038	
" " neutral fat alone (D - E)	0.004	
" " fatty acids freed from soap (F)	0.307	
" " soap (G) = (F $\times$ 1.1)	0.338	
" " total fat in sample of stool (C + D + G)	0.442	

From these figures the per cent of the fat in the dried stool and the percentage of the various constituents of the total fat can readily be calculated.

$$\text{Per cent of fat in dried stool} \left( \frac{0.442}{1.837} \right) = 24.1 \text{ per cent}$$

$$\text{Stool fat as unsaponifiable} \left( \frac{0.038}{0.442} \right) = 8.6$$

$$\text{" " " neutral fat} \left( \frac{0.004}{0.442} \right) = 0.9$$

$$\text{" " " free fatty acids} \left( \frac{0.062}{0.442} \right) = 14.0$$

$$\text{" " " soap} \left( \frac{0.338}{0.442} \right) = 76.5$$

A somewhat abbreviated procedure can be used if it is not desired to make determinations of free fatty acid and soap, but only of split fat as a whole. In this case, the concentrated HCl is added at the beginning, necessitating only one extraction for removal of all lipids. The split fat is then determined by titration of the fatty acids. It is essential that the last traces of HCl be washed out with water before the fatty acids are titrated. After the titration the fat is saponified and the unsaponifiable fraction extracted as described above.

It is a disputed question whether any significant information can be gained from a separate estimation of the free fatty acid and soap. It has been maintained that the proportion of free

fatty acid to soap in the feces is determined merely by the amount of base available in the intestine to combine with such fatty acid as may be unabsorbed. Keller (8), for example, found that there was very little constancy in the proportion of alkali soaps to those of alkaline earths, and his views have been followed by Folin and Wentworth (6) and others (9). On the other hand, it seems possible that the soaps of the stool may indicate something more than this. The formation of insoluble alkaline earth soaps in the intestine has been regarded by Bosworth, Bowditch, and Giblin (10) as a factor interfering to some extent with fat absorption, a view which finds some support in our own work (11)

#### EXPERIMENTAL

We have tested the analytical procedure outlined above by adding to normal and to fat-free specimens of stool known amounts of neutral fat (olive oil), free fatty acid (oleic acid), soap (calcium stearate), and unsaponifiable matter (from feces) to stools. The added material was thoroughly mixed by adding a fat solvent which was then evaporated off. It was found that such added material could be recovered quantitatively in the appropriate fraction with an error rarely exceeding 1 per cent.

Since it has been claimed, particularly by Fowweather (12), that analyses of the fat partition carried out on dried stools are inaccurate, and that the wet method should always be employed, it seemed desirable to make some comparisons by these two methods. The stools on which these analyses were run were kept covered with 50 per cent alcohol until the collection was complete,<sup>3</sup> they were then thoroughly mixed, and while the material was still pasty in consistency, samples were removed for wet analysis. The remaining portion of the stool was subjected to our usual drying procedure which consisted in evaporating to dryness at room temperature, followed by pulverizing, after which the last traces of moisture were removed in a desiccator. The samples of dried stool were kept in tightly stoppered bottles and analyzed within 2 weeks after removal from the desiccator. Results are given in Table I, in which each figure represents an average of analyses made in triplicate.

<sup>3</sup> Less than 1 day in the case of Specimens M-1 and D, 2 weeks in the case of Specimen M-2

It appears that no significant alteration in the composition of the stool results from drying in the air with the procedure we have employed.

#### DISCUSSION

We wish to take this opportunity of pointing out how previous observers, following the conventional procedure of expressing the various lipid fractions in percentages of the fecal fat, rather than in absolute quantities, and failing to appreciate the presence of much unsaponifiable matter in the "neutral fat" fraction, have been led to quite erroneous conclusions in regard to fat splitting. Table II

TABLE I  
*Determination of Fat Partition in Wet and Dry Stools*

Stool specimen No	Method	Fat in total solids of stool	Partition of stool fat		
			Neutral fat + unsaponifiable	Free fatty acids	Soap
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
M-1	Wet	71.7	60.9	18.5	20.6
	Dry	72.7	60.9	19.1	20.1
D	Wet	69.6	34.3	19.6	46.0
	Dry	70.9	33.8	18.1	48.1
M-2	Wet	56.6	16.1	24.2	59.7
	Dry	57.0	17.0	20.5	62.5

gives typical data on the fat partition of the stools of infants fed on breast milk and on cow's milk.

On cow's milk the absorption of fat is less complete and correspondingly more fat is found in the stool; yet in spite of this, the proportion of unsplit "neutral fat" in the stools is never as high as on breast milk, and the high proportion of split fat in the form of soap has always occasioned comment. When, however, the fecal fat is expressed in absolute amounts, as we have done in the second half of Table II, the quantities of "neutral fat" and free fatty acid prove to be practically identical in the two sets of experiments, the increase in fecal fat on cow's milk being due almost entirely to unabsorbed soap. The peculiar constancy of the "neutral fat" fraction led us to suspect that this might consist largely of unsaponifiable material, and this suspicion was con-



firmed when we determined its content in the fecal fat. Analyses reported elsewhere (11) revealed that under normal conditions the "neutral fat" fraction is virtually all unsaponifiable matter. It is worthy of comment that the percentage of unsaponifiable matter in the "neutral fat" fraction of human feces appears to be considerably higher than has been reported in dogs. In these animals, Sperry (14) reported that approximately 75 per cent of the "neutral fat" fraction was unsaponifiable, whereas in normal

TABLE II  
*Fat Partition of Stools of Infants (Data of Holt, Courtney, and Fales (13))*

Diet	Fecal fat per day	Fat partition					
		Per cent of fecal fat			Gm per day		
		Neu-tral fat	Free fatty acids	Soap	Neu-tral fat	Free fatty acids	Soap
	<i>gm</i>						
Breast milk	1 15	19 7	27.7	52 6	0 23	0 32	0 60
Cow's milk . . . .	2 39	9 6	14 4	76 0	0 23	0 34	1.82

TABLE III  
*Fecal Lipids of Normal Infants*

Diet	Total lipids of feces per day	Partition of fecal lipids per day			
		Un-sapon-ifiable	Neu-tral fat	Free fatty acids	Soap
	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
Skim milk + breast milk fat. . . .	1 85	0 21	0 02	0 58	1 04
" " + cow's milk fat	2 88	0 22	0 03	0 54	2 09

infants we have found it to be at least 90 per cent. Only in pathological states and with certain very poorly absorbed fats does the true neutral fat constitute an appreciable proportion of the feces. Table III presents typical analyses which we have made on normal infants receiving breast milk fat and cow's milk fat.

#### SUMMARY

1. A method of determining the partition of fecal lipids is presented, which avoids certain difficulties inherent in previous

methods, and permits the determination of unsaponifiable matter, neutral fat, fatty acid, and soap in a single sample.

2. Reasons are given for abandoning the conventional practice of expressing the partition in percentages of fecal fat. A more accurate picture of the facts can be obtained by expressing them in absolute quantities.

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# THE INFLUENCE OF FEEDING AMINO ACIDS AND OTHER COMPOUNDS ON THE EXCRETION OF CREATINE AND CREATININE\*

By MEYER BODANSKY

WITH THE TECHNICAL ASSISTANCE OF VIRGINIA B. DUFF

(From the Department of Pathological Chemistry, School of Medicine, University of Texas, and the John Sealy Hospital, Galveston)

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Few problems in biochemistry have proved more perplexing than the question of the origin of creatine. Beginning with the early studies which attempted to link this compound with some specific metabolic rôle, interest in the problem has been more or less well sustained, and in recent years added stimulus has been derived through the newer developments in our conception of muscle metabolism. The subject has also acquired some importance clinically, following the demonstration by Brand and associates (1) that in cases of progressive muscular dystrophy the ingestion of glycine produces a marked increase in creatine excretion, and the further efforts by numerous workers to establish the therapeutic value of this and other amino acids in muscle disease.

To the discordant data in the literature prior to 1928, when Hunter (2) prepared his excellent monograph, data have been added since, which far from clarifying the situation have contributed only to its confusion. The literature since 1928 has been critically summarized in two reviews by Rose (3).

Beard and Barnes (4) have reported large increases in the daily excretion of total creatinine in the urine of adult rats following the administration of various amino acids, proteins, and other compounds. Many of these, though chemically unrelated to creatine, when fed to young rats, produced a marked increase in the concentration of this constituent in the muscle. The authors therefore suggest that these substances stimulate creatine-creatinine meta-

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bolism, in some way other than by specific dynamic action, with a resulting increased formation of creatine in the muscles and elimination of creatinine in the urine

Because of the importance attached at present to the problem of creatine precursors, it seemed desirable to verify some of the results obtained by Beard and Barnes (4). The work has been extended to include several other substances, the relation of which to creatine metabolism has been the subject of dispute. The compounds studied and their sources were as follows: *dl*-alanine (Eastman), *d*-arginine (Hoffmann-La Roche), *l*-aspartic acid and *d*-glutamic acid (Eastman), *l*-cystine (Hoffmann-La Roche), glycine (Calco, Dow), *l*-tryrosine (Pfanstiehl), *l*-histidine monohydrochloride, choline hydrochloride, betaine hydrochloride, and sarcosine (Hoffmann-La Roche), guanidine hydrochloride, guanidine acetate, and guanidine carbonate (Eastman), guanidineacetic acid (glycocyamine) and methylguanidine hydrochloride (Hoffmann-La Roche), creatone, prepared according to the method of Greenwald (5), creatine hydrate (Eastman), several times recrystallized.

#### EXPERIMENTAL

The data in this paper are based on metabolism studies performed on seventeen young adult rats (ten males, seven females), with an average weight of 200 gm. Each rat was kept in a separate metabolism cage which rested part way in a large glass funnel. The floor of the cage was made of  $\frac{3}{4}$  inch wire mesh. Food particles and feces were prevented from falling into the urine receptacle by a wire screen at the bottom of the funnel. The animals were maintained on a creatine-free diet similar to that employed by Chanutin and Silvette (6) and Beard and Barnes (4). Analysis of the urine was carried out on 48 hour specimens. With ordinary precautions in collection, rinsing down the sides of the funnels, and periodic removal of accumulated material on the wire screen, the results, as regards the excretion of total nitrogen, creatine, and creatinine, were remarkably consistent. All substances tested were administered by stomach tube (French catheter No. 8 or 10 inserted through a hollow metal tube used to keep the jaws apart). Following several 48 hour control periods, during which the rats became accustomed to the new environment,

the test substance was given at the beginning of a new 48 hour period. The experimental period was followed by a similar control or rest interval, after which the rat was ready for the next trial, either with the same or some other compound. Only exceptionally was there any apparent advantage in extending the rest period

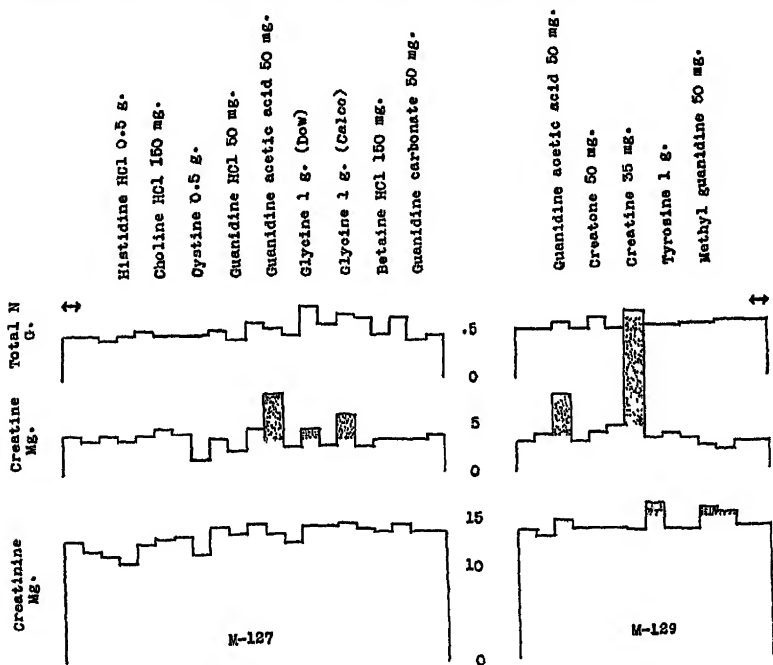


FIG. 1. The effect of various compounds on the urinary excretion of total nitrogen, creatine, and creatinine in Rats M-127 and M-129. The levels of excretion as represented are for 48 hour periods. The compounds tested were administered at the beginning of one period, following which there was a control period of 48 hours. The length of either of the two double arrows is equivalent to a 48 hour period.

beyond 48 hours. Indeed the effects of most compounds were dissipated within the first 24 hours, with a return to basal levels of excretion during the succeeding 24 hours.

None of the animals in this group was adversely affected by the experimental procedures and all continued to gain weight at approximately normal rate. The average duration of the experi-

ments was 30 days, though with several of the rats the experiments were continued as long as 60 days. Accompanying the increase in weight there was a gradual increase in the basal daily excretion of preformed creatinine and total nitrogen

The curves in Fig. 1, in addition to representing a number of typical results, illustrate the general conduct of the experiments. One set of curves is based on data obtained on Rat M-127 during the interval February 20 to April 3, and the other on those of Rat M-129 for the period March 10 to April 7. The length of each double arrow represents a 48 hour interval.

The various compounds employed in this study were tested for chromogenic properties by autoclaving with alkaline picrate, as in the estimation of creatine plus creatinine. Essentially negative results were obtained with all the amino acids, in amounts of 100 to 200 mg., save histidine, which produced a definite, though from the standpoint of this investigation, a negligible, effect (100 mg.  $\approx$  < 0.01 mg. of creatinine). Absolutely negative reactions were noted with sarcosine, betaine, choline, creatone, methylguanidine, and the guanidine salts (hydrochloride, carbonate, and acetate). Guanidineacetic acid reacted positively, the color effect produced by 100 mg. being equivalent to approximately 4 mg. of creatinine, a fact which must be taken into account in interpreting the metabolic data with this compound.

### *Results*

The results are summarized in Table I. In evaluating them a safe criterion is to consider a variation in the 48 hour excretion of  $\pm 1$  mg. to be of no significance. The writer holds the view that creatinuria resulting from the administration of a given substance is not necessarily proof of the production of extra creatine. At best such evidence is only presumptive. In this light the present report is regarded primarily as a study of specific and non-specific creatinuria and not as an investigation of the precursors of creatine. Yet even at this stage it is perhaps reasonable to expect, under the conditions of the experiment, that if a given substance is actually a precursor of creatine, the conversion would be sufficient to yield a significant extra excretion of creatine (or creatinine). Conclusions cannot be drawn from minor changes which may conceivably result from a variety of factors, such as diuretic effects,

non-specific metabolic stimulation, and toxic influences, at least in the case of certain compounds.

*dl-Alanine*—In the experiments of Beard and Barnes (4), rats (200 to 300 gm.) receiving 1.5 gm. of *dl*-alanine showed an average increase of 28 per cent in creatinine output. These workers also found an increase in muscle creatine in young rats receiving this amino acid. In cases of progressive muscle dystrophy receiving alanine, Brand *et al.* (1) observed a slightly increased creatine output. In rabbit heart perfusion experiments, Decherd, Herrmann, and Davis (7) found that of various amino acids alanine alone seemed able to maintain and even enrich the creatine content of the heart muscle. Working with rats, MacKay and Barnes (8) observed that the addition of glycine or alanine to the diet definitely raised the creatine content of the myocardium. As indicated by the data in Table I, the administration of 1 to 2 gm. of alanine produced no significant change in two experiments, an increase of 1 to 1.9 mg. in five others, and of 2 to 4 mg. in two experiments. In four of a total of eleven trials, the output of preformed creatinine exceeded the basal level by an average of about 10 per cent. In the remainder the creatinine excretion was unaffected. From these results it would seem that while alanine produces a somewhat inconstant effect on creatine (and creatinine) elimination, the change is not such as to indicate that it is necessarily related to increased creatine production.

*d-Arginine*—Despite their close chemical relationship, evidence for a direct transformation of arginine into creatine is lacking. The prolonged administration of relatively large amounts of arginine to human subjects produced no increase in the excretion of either creatine or creatinine in the experiments of Hyde and Rose (9). Confirmatory results were obtained in dogs by Grant, Christman, and Lewis (10). On the other hand, Shapiro and Zwarenstein (11) reported an increase in urinary creatinine in rabbits following the injection of arginine. Beard and Barnes (4) recorded an average creatinine increase of 26.8 per cent in their rats, following the administration of a 1 gm. dose of the amino acid. No significant changes were observed in any of our experiments (dosages of 0.1 to 1.5 gm.).

*l-Aspartic Acid*—This amino acid, contrary to the observations of Beard and Barnes (4), produced no effect on the elimination of creatine or creatinine.



TABLE I

*Effect of Feeding Rats Certain Amino Acids and Other Compounds on 48 Hour Excretion of Creatine*

The number of experiments with each compound is given by the figures in the third to the seventh columns.

Compound	Amount	Decreases of 1-2 mg	No changes, ±1 mg	Increases of			mg.
				1-1.9 mg	2-4 mg	> 4 mg	
<i>dl</i> -Alanine	0.5 gm		2				
	1 "		2				
	2×1 "			2			
<i>d</i> -Arginine	0.1 "		2	1	2		
	3×50 mg.		2				
	0.5 gm.		2				
	3×0.5 "		1				
<i>L</i> -Aspartic acid	0.2 "		1				
	0.5 "		1				
	3×0.5 "		1				
<i>L</i> -Cystine	0.5 "	2	3				
	2×0.5 "		2				
	3×0.5 "		2				
<i>d</i> -Glutamic acid	0.2 "		2				
	0.5 "		2				
	3×0.5 "		2				
Glycine (Calco)	0.5 "			6	2	1	5
	1 "			2	8	3	4.1, 4.5, 5.1
" (Dow)	1 "		2	1	2	1	4.5
Histidine mono- hydrochloride	0.5 "		1				
	2×0.5 "	1	2	1			
	3×0.5 "		2				
<i>L</i> -Tyrosine	0.5 "		2				
	2×0.5 "		1				
Choline hydro- chloride	150 mg		5	1			
Betaine hydro- chloride	150 "	1	5		1		
Sarcosine	150 "		1				
	0.5 gm		3				
	2×1 "		1				
Guanidine hydro- chloride	50 mg.		3	1			
Guanidine car- bonate	50 "		2				
	80 "		3				

TABLE I—*Concluded*

Compound	Amount	Decreases of 1-3 mg	No changes, ±1 mg	Increases of			mg
				1-1.9 mg	2-4 mg	>4 mg	
Guanidine acetate	50 mg.		2	1			
	100 "	2	5				
Guanidineacetic acid	50 "				5	6	4 2, 4.5, 5, 8 2, 9, 9 5
	60 "					6	8, 8 1, 9 6, 10, 13, 15
	100 "					4	6, 8, 14, 19 6
Methylguanidine hydrochloride	50 "		2	1			
	100 "		1	1			
Creatine	35 "					2	12, 14 7
	50 "					3	16, 20, 29
	60 "					4	22, 32, 36, 43
	120 "					2	48, 61
Creatone	50 "		4				
	2×50 "		3	1			

*l-Cystine*—In experiments on dogs, Harding and Young (12) found that the administration of cystine increased the output of creatine. Gross and Steenbock (13) observed the same effect in hogs, but nevertheless did not accept the conclusion of Harding and Young that cystine is a precursor of creatine. Doses of 1.5 gm. produced an average increase of 28.1 per cent in the creatinine output in the experiments of Beard and Barnes (4). In contrast, the writer observed a slightly diminished output of creatine and creatinine in two experiments and no significant change in seven others (dosages 0.5 to 1.5 gm.).

*d-Glutamic Acid*—In contrast to the data of Beard and Barnes (4) the administration of glutamic acid produced no demonstrable effect on the excretion of either creatine or creatinine.

*Glycine*—With one exception in which the preformed creatinine was increased by 2 mg. for the 48 hour period, the creatinine output was unaffected by the administration of glycine. This is in agreement with the observations on human subjects by Brand and associates (1). With one commercial preparation (Calco) the output of creatine was invariably increased, while with a second

preparation (Dow) this effect seemed to be somewhat less pronounced, as indicated by the data in Table I. This observation led us to consider the possibility that the increased output of creatine, following the administration of glycine, may be due to the presence even in highly purified commercial preparations of an adventitious substance, such as guanidineacetic acid. By an adaptation of Weber's procedure (14) for the estimation of guanidineacetic acid in urine, tests were performed on the Calco and Dow products, as well as on those of Merck and Eastman. The results were uniformly negative.

*l-Histidine*—An increased output of total creatinine, following the administration of histidine, has been reported by Abderhalden and Buadze (15) in experiments on dogs, by Shapiro and Zwarenstein (11) in rabbits, and by Beard and Barnes (4) in rats. Although in one of our experiments, feeding histidine resulted in a slight increase in creatine elimination, while in another there was a delayed output of 1.5 mg. of extra creatinine, the evidence taken as a whole is opposed to the observations reported by the various investigators just mentioned, but is in agreement with a considerable accumulation of earlier data, cited by Hunter (2), as well as with more recent observations of Brand and coworkers (1) and Schumann (16).

*l-Tyrosine*—Experiments with this amino acid were included because of the statement of Beard and Barnes (4) that feeding of 1.5 gm. increased the creatinine output approximately 30 per cent. Our results with doses of 0.5 to 1 gm. were entirely negative.

*Choline, Betaine, and Sarcosine*—The chemical relationship of these substances to creatine has stimulated considerable speculation concerning their metabolic significance as intermediates. Choline hydrochloride in doses of 150 mg. produced a definite, though slight, increase in only one of six experiments. Betaine hydrochloride in similar dosage caused a rise of 2.2 mg. in one trial, no change in five, and a decrease of 1.6 mg. in another. With sarcosine the results were all negative.

*Guanidine and Methylguanidine*—Guanidine administered as the hydrochloride, carbonate, and acetate in doses of 50 to 100 mg. produced no significant effects in fifteen trials; small increases were observed in two experiments and comparable decreases in the output of creatine in two others. A dose of 150 mg. of guanidine

carbonate given to one rat proved fatal. Methylguanidine hydrochloride produced an appreciable increase in the output of creatine in two out of five experiments; an increase in the output of creatinine was observed in a third experiment. However, no clear significance can be attached to these results. If methylguanidine were truly a precursor of creatine, this would have been reflected by more striking changes in the composition of the urine.

*Guanidineacetic Acid*—The output of creatine was increased from 2.8 to 9.6 mg., following the administration of 50 mg. of guanidineacetic acid (glycocyamine). Doses of 100 mg. produced increases of 4 to 19.6 mg. It will be recalled that the preparation used in this work reacted with alkaline picrate. However, even if all of the guanidineacetic acid had been excreted unchanged, the analytical results should not have exceeded the basal excretion of creatine by more than 2 mg. for the 50 mg. dose, or 4 mg. for the 100 mg. dose. The majority of data presented therefore indicate the probable conversion of guanidineacetic acid into creatine. While this view is widely accepted and is supported by a considerable number of data, it is nevertheless felt that final proof must rest on more direct evidence than the increased urinary creatine output.

*Creatone*—This oxidation product of creatine, first described by Baumann and Ingvaldsen (17), was prepared according to the method of Greenwald (5). No effect was observed with 50 mg. doses. The administration of 100 mg. increased the creatine output less than 2 mg. in one experiment and produced no significant change in three others.

*Creatine*—Creatine hydrate was given in doses equivalent to 35 to 120 mg. of creatine. The amount recovered in the urine within 48 hours varied from about 30 to 80 per cent

#### SUMMARY

Arginine, aspartic acid, glutamic acid, cystine, histidine, and tyrosine, administered in comparatively large doses by stomach tube, produced no effect on the excretion of creatine or creatinine in rats.

The administration of glycine did not influence the excretion of creatinine, but definitely increased the output of creatine. Similar, though less striking or constant changes, were obtained with alanine.

Choline, betaine, sarcosine, and the salts of guanidine did not affect the output of either creatine or creatinine. Methylguanidine increased somewhat the elimination of creatine in two out of five experiments, but no special significance is attached to this observation. The increased elimination of creatine following the administration of guanidineacetic acid suggests the probable conversion of the latter into creatine. However, the point is emphasized that an increased elimination of creatine under these conditions is, at best, only presumptive evidence of extra creatine formation.

Creatine given in doses varying from 35 to 120 mg. was recovered in the urine within 48 hours in amounts varying from about 30 to 80 per cent. Creatone, an oxidation product of creatine, produced no significant change in the output of either creatine or creatinine.

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## STUDIES ON BIOLOGICAL OXIDATIONS

### V. COPPER AND HEMOCHROMOGENS AS CATALYSTS FOR THE OXIDATION OF ASCORBIC ACID. THE MECHANISM OF THE OXIDATION

BY E. S. GUZMAN BARRON, R. H. DeMEIO,\* AND  
FRIEDRICH KLEMPERER

*(From the Lasker Foundation for Medical Research and the Department of  
Medicine of the University of Chicago, Chicago)*

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Since the identification of ascorbic acid with vitamin C, the physiological importance of this substance has been increasing considerably. Ascorbic acid seems to be, by virtue of its high reducing power, one of the systems which maintain the oxidation-reduction equilibrium necessary for the harmonious interreactions of cellular oxidations and reductions. Unfortunately, the literature on this problem is full of contradictory statements. Thus, it has been taken for granted without adequate experimental evidence that ascorbic acid "avidly takes up oxygen" (Harris (1)) and is destroyed by aeration. With reference to the thermodynamic reversibility of this system, we are faced with reports (Laki (2), Karrer, Schwarzenbach, and Schopp (3), and Green (4)) that the system is thermodynamically irreversible, and with others (Georgescu (5), Borsook and Keighley (6), Wurmser and de Loureiro (7), and Fruton (8)) that it is thermodynamically reversible. Strangely, even among those who maintain the latter opinion, there is no agreement in their reported data.

A prerequisite to a study of the rôle of ascorbic acid in biological oxidations must be, as Clark emphasizes in his admirable review (9), "first, the comprehension of components." We, therefore, decided to study first the so called autoxidation of ascorbic acid in carefully prepared buffer solutions and proceeded then to a study of the catalysts which accelerate the oxidation, the mechanism of this reaction, and its chemical reversibility.

\* Fellow of the Rockefeller Foundation.

## EXPERIMENTAL

We wish to emphasize that extreme care was taken to avoid contamination of our solutions by heavy metals. The water used for the preparation of buffers and for the final washing of the glassware was twice distilled in a quartz distillation apparatus after having been thrice distilled at the laboratory. All the chemicals used for the preparation of buffers and those tested for their catalytic power were obtained from Kahlbaum. The ascorbic acid obtained from Merck (natural) was recrystallized. The hemin was prepared at the laboratory and thrice recrystallized. All reagents before being used were tested for iron with thiocyanate and for copper with Kolthoff's method (10). All solutions were kept either in porcelain flasks or in paraffined bottles. The Warburg vessels, made of Pyrex glass, were boiled in cleaning solution and washed with quartz-distilled water at the end of each experiment. The ends of the manometers were cleaned in the same way. Stop-cock grease was avoided by sealing the vessels to their respective manometers with paraffin. The oxidation of ascorbic acid was determined by measurement of the oxygen consumption by the well known Warburg method, the ascorbic acid being kept in the side arm and dropped into the main vessel when temperature equilibrium was reached. The temperature of all experiments, unless otherwise stated, was 25°. The amount of ascorbic acid used in each vessel was 0.02 mm (dissolved in 0.2 cc. of water). The pH values of the buffer plus ascorbic acid were determined with the glass electrode up to pH 9; above pH 9, with the hydrogen electrode. The hydrogen ion concentrations of the solutions were controlled as follows: HCl 0.1 M, Sørensen's citrate and borate mixtures, Clark's  $\text{KH}_2\text{PO}_4\text{-NaOH}$  mixtures, and Kolthoff and Vleeschauwer's  $\text{Na}_2\text{HPO}_4\text{-NaOH}$  mixtures (Clark (11)).

*So Called Autoxidation of Ascorbic Acid*

The autoxidizability of ascorbic acid has been taken for granted, probably because of its high reducing power. When great care was taken to use solutions not contaminated with copper, the oxidation of ascorbic acid by atmospheric oxygen at 25° failed to take place with measurable speed (the oxygen consumption being measured for 1 hour) in experiments performed at different

pH values up to pH 7.60 (Fig 1). It was found that ascorbic acid is autoxidizable only in alkaline solutions, the rate of oxidation increasing considerably as the alkalinity increases from pH 8 to pH 10. At pH 8.30 the oxygen consumed in 1 hour was 8.3 c.mm.; at pH 8.66, it was 36 c.mm.; and at pH 9.66, it was 178 c.mm., this last increase of 1 pH (from 8.66 to 9.66) having raised the rate of oxidation 4.9 times. This rapid autoxidation of

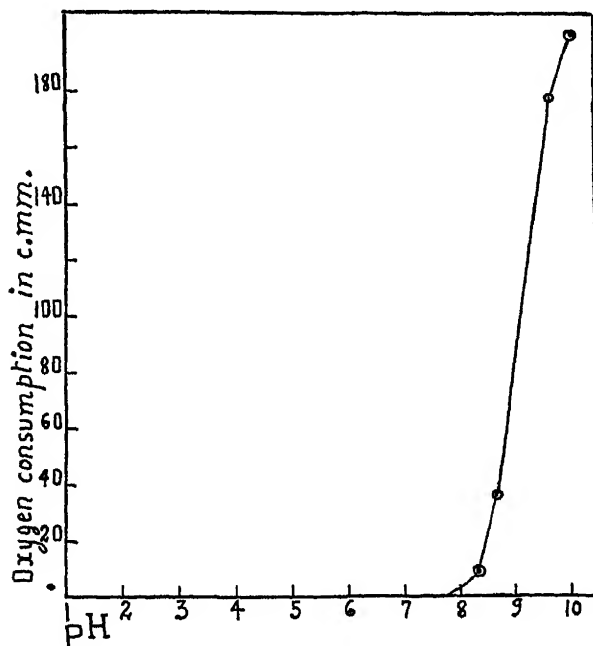


FIG. 1. The autoxidation of ascorbic acid at different hydrogen ion concentrations. Abscissa, pH; ordinate, oxygen consumption in 60 minutes (c.mm.);  $T$ ,  $25^{\circ}$ ; amount of ascorbic acid, 0.02 mm.

ascorbic acid in alkaline solutions, already observed by the Birmingham School of Chemistry (12) and by Karrer, Salomon, Morf, and Schopp (13), proceeds after 1 atom of oxygen has been used per molecule of ascorbic acid (as can be seen in Fig. 2), and ends with the formation of oxalic acid and *l*-threonic acid. As pointed out by Euler, Myrback, and Larsson (14), the addition of KCN (0.001 M per liter) not only failed to inhibit the rate of oxidation



but increased it slightly because of a higher reactivity of the cyanhydrin formed. From these experiments we must therefore conclude that, contrary to statements frequently made, ascorbic acid is not autoxidizable at physiologic hydrogen ion concentrations.

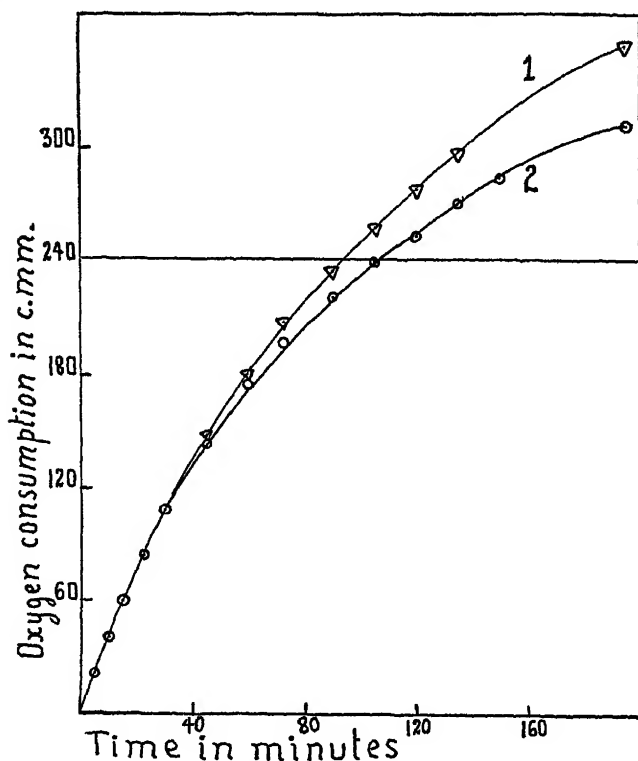


FIG. 2 The autoxidation of ascorbic acid in borate buffer. pH, 9.01;  $T$ ,  $25^{\circ}$ . Curve 1 represents autoxidation in the presence of  $0.001\text{ }M$  KCN; Curve 2, autoxidation of ascorbic acid alone. The black line represents the amount of oxygen which would be consumed by the oxidation of ascorbic acid to dehydroascorbic acid.

#### *Copper As Catalyst for Oxidation of Ascorbic Acid in Atmospheric Oxygen*

The destruction of vitamin C in foods by copper was observed as early as 1921 by Hess and Unger (15). Euler, Myrback, and Larsson (14) confirmed these findings and studied the effect of

increased concentrations of copper on the rate of oxidation of ascorbic acid. The enormous oxygen uptake shown by the controls without copper invalidates their results. The oxidation of ascorbic acid in acid solutions up to pH 6.60, with copper as

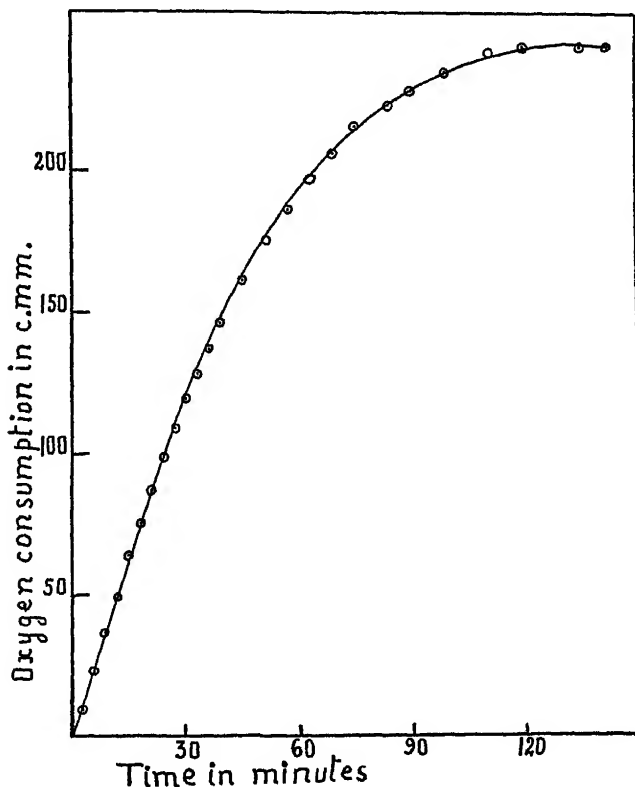


FIG 3 The oxidation of ascorbic acid by atmospheric oxygen with copper as catalyst pH, 5.15;  $T$ ,  $25^{\circ}$ ; amount of cupric chloride, 0.0002 mm; amount of ascorbic acid, 0.02 mm; calculated oxygen consumption, 244.5 c.mm.; found, 244 c.mm.

catalyst (0.0002 mm of  $\text{CuCl}_2$ ), requires 1 atom of oxygen per molecule of ascorbic acid, just as is the case in the oxidation by iodine or by Clark's 2,6-dichlorophenol indophenol. In Fig. 3 is reported an experiment performed at pH 5.15. The oxidation was

finished in 2 hours with an oxygen uptake of 244 c.mm., which corresponds to 1 atom of oxygen per molecule (amount of ascorbic acid, 0.02 mm). Experiments such as this were performed at different pH values, from 4.17 to 6.60, with the same results.

*Effect of Varying Concentrations of Copper*—The catalytic effect of copper was noticed with the addition of such a minute quantity of copper as 46 micrograms per liter (0.000727 mm of  $\text{CuCl}_2$  per liter). This extraordinary catalytic power of copper probably explains the often repeated reports about the autoxidizability of ascorbic acid. In these experiments the pH was kept constant (3.17) as well as the ascorbic acid (0.02 mm), while the concen-

TABLE I

*Effect of Copper Concentration on Rate of Oxidation of Ascorbic Acid*

pH, 3.17 (citrate buffer); amount of ascorbic acid, 0.02 mm;  $T$ , 25°; volume of fluid, 2.75 cc.

Amount of $\text{CuCl}_2$	Concentration per liter	Time required for half oxidation of ascorbic acid
mm	mm	min
0.000002	0.000727	1748*
0.00002	0.00727	181.0
0.00005	0.01818	85.0
0.0002	0.0727	49.5
0.0005	0.1818	19.0
0.002	0.727	10.3
0.005	1.818	8.5
0.01	3.6	6.0

\* Calculated value; 8.90 mm. in 120 minutes.

tration of copper was varied. Since the rate of oxidation was constant (zero order reaction) until about half of the substrate was oxidized, the time required to reach half oxidation was determined. With an increase of 1:10 in the concentration of copper (i.e., from  $2 \times 10^{-6}$  to  $2 \times 10^{-5}$  milliatom) the rate of oxidation of ascorbic acid increased also 1:10; with an increase of 1:100 in the concentration of copper, the rate of oxidation increased 1:35; with an increase of 1:1000, the rate of oxidation increased 1:174. (The ratio of catalyst to ascorbic acid was in the last case 1:10.) From here on, an increase in the concentration of the catalyst did not affect appreciably the rate of oxidation (Table I).

*Effect of Hydrogen Ion Concentration*—To determine the influ-

ence of the hydrogen ion concentration on the catalytic power of copper, a range of pH values from 1.07 to 10.16 was covered. The amount of ascorbic acid (0.02 mM) and the amount of catalyst (0.0002 mM of  $\text{CuCl}_2$ ) were kept constant. The time required to reach half oxidation was determined, there being in every experiment controls without copper. When the pH value at which autoxidation starts (pH 7.6) was reached, the amount of oxygen consumed by the control was subtracted from the amount consumed in the presence of the catalyst. At pH 1.07 the rate of oxidation was so slow that half oxidation would have been reached only at the end of 367 minutes. From this point the rate of oxidation increased very rapidly, so that at pH 2.24 half oxidation was

TABLE II

*Effect of Hydrogen Ion Concentration on Catalytic Power in Oxidation of Ascorbic Acid by Atmospheric Oxygen*

Amount of  $\text{CuCl}_2$ , 0.0002 mM, amount of ascorbic acid, 0.02 mM;  $T$ , 25°.

pH	Time required for half oxidation	pH	Time required for half oxidation
	min		min
1.07	367 (Calculated)	6.00	15.0
2.24	60	6.75	12.0
2.95	53.2	6.95	10.3
3.17	51.1	7.20	12.0
4.05	36.0	8.30	30.1
4.29	30.5	9.01	42.3
5.10	22.5	10.16	58.5

reached in 60 minutes, a 6-fold increase in the rate of oxidation. From this pH on, the rate increased slowly, reaching its maximum speed at pH 6.95, where half oxidation was produced at the end of 10.3 minutes (Table II).

*Effect of Manganese, Iron, Nickel, Cobalt, and Calcium on Oxidation of Ascorbic Acid*

Euler, Myrback, and Larsson (14) reported that manganese, iron, nickel, and calcium increased the oxidation of ascorbic acid; a similar claim was made for Ringer's solution. Cobalt, on the other hand, they reported, inhibited the oxidation. We performed a great number of experiments at pH values of 4.15 and

6.28 with the following metallic salts: manganese sulfate, nickel chloride, ferrous ammonium sulfate, ferric chloride, cobalt sulfate, and calcium chloride, which were used in amounts varying from 0.004 mM to 0.01 mM. Ascorbic acid was used in the usual amount, 0.02 mM. None of these metals acted as catalyst for the oxidation of ascorbic acid, as there was no oxygen consumption after the addition. Since the results were consistently negative, the data of these experiments are omitted.

#### *Catalytic Power of Hemochromogens*

It has been shown by Tillmans, Hirsch, and Hirsch (16) that reversible dyes of high oxidation potential (indophenols) can be utilized for the determination of ascorbic acid in acid solutions provided that the titration is carried out rapidly, as the rate of reoxidation of these dyes is quite low (Barron (17)). It is obvious that a number of reversible dyes possessing the proper oxidation-reduction potential could act as catalysts for the oxidation of ascorbic acid in atmospheric oxygen if the rate of reoxidation of the reduced dye is not low. By the same reasoning, hemochromogens, as reversible systems, could also act as catalysts if their potential were such as to make the progress of the oxidation a thermodynamic possibility.

It is already known that iron becomes a powerful catalyst for a number of oxidations of biologic interest when it combines with porphyrin to form hemin. Moreover, the catalytic power of hemin is enhanced when it combines with a number of nitrogenous derivatives to form hemochromogens, this increased catalytic power being probably due to an increase in the oxidation potential of hemin when it is transformed into hemochromogen. Hemin and the hemochromogens of nicotine, pyridine, and pilocarpine were tested for their catalytic power at pH 6.39. The amount of hemin was 0.00015 mM; the amount of nicotine and pilocarpine, 3.55 mM; that of pyridine twice as much. (None of these nitrogenous bases produces the oxidation of ascorbic acid when used alone.) As can be seen in Fig. 4, the three hemochromogens acted as catalysts for the oxidation of ascorbic acid in the following decreasing order: nicotine > pyridine > pilocarpine. Hemin had no catalytic power. It is interesting to note that the catalytic power of the hemochromogens increased as their oxidation-

reduction potentials became more positive. For, according to the measurements of Barron and Hastings, as yet unpublished, the  $E'_0$  of these hemochromogens is, at pH 9.16,  $-0.005$  volt for nicotine,  $-0.050$  volt for pyridine,  $-0.156$  volt for pilocarpine,  $-0.240$  volt for hemin. When nicotine-hemochromogen was used as catalyst, the series of reactions which occurred during the

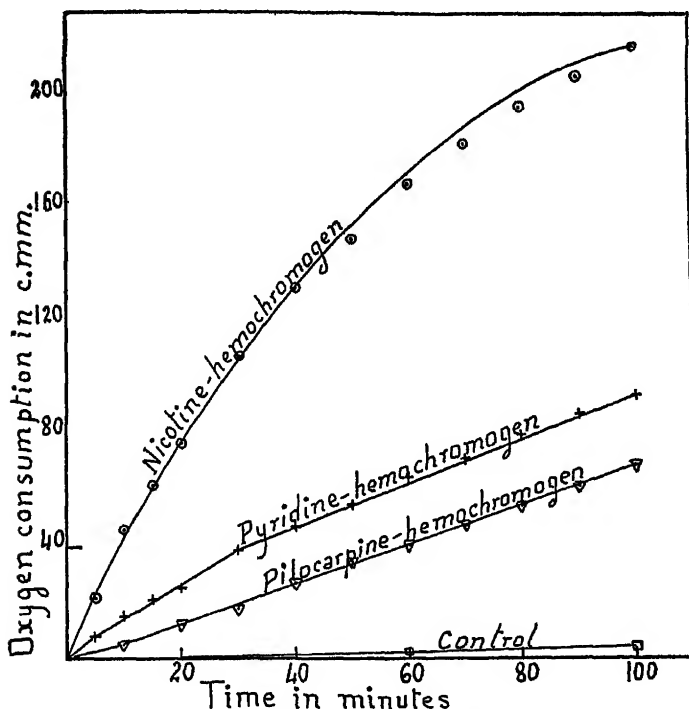


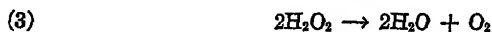
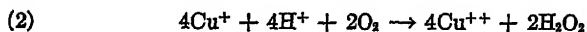
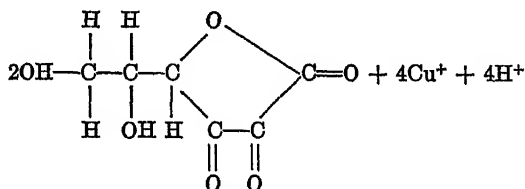
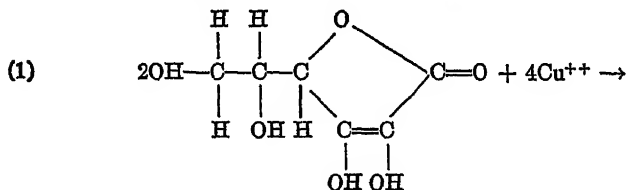
Fig 4 The catalytic effect of hemochromogens on the oxidation of ascorbic acid by atmospheric oxygen pH, 6.39;  $T$ ,  $25^{\circ}$ ; amount of ascorbic acid, 0.02 mm; amount of hemin in the hemochromogens, 0.00015 mm.

oxidation of ascorbic acid could be followed spectroscopically. As soon as the oxidized nicotine-hemochromogen came in contact with the buffered solution containing ascorbic acid, the formation of reduced nicotine-hemochromogen could be detected by the change in color and the appearance of the two typical bands of the reduced compound ( $5575 \text{ \AA.}$  and  $5265 \text{ \AA.}$ ). After a thorough

shaking, the oxidized hemochromogen with its typical olive-green color reappeared. The catalytic power of nicotine-hemochromogen is only 0.43 of the catalytic power of copper. Thus, 0.00015 mm of nicotine-hemochromogen consumed 46.5 c.mm. of oxygen in 12 minutes, whereas the same amount of copper consumed 108.6 c.mm. Of course, since the dissociation constant of nicotine-hemochromogen is not yet accurately known, the *actual* concentration of hemochromogen in these experiments cannot be definitely stated.

#### *Mechanism of Oxidation of Ascorbic Acid*

It can be postulated that the oxidation of ascorbic acid by atmospheric oxygen with either copper or hemochromogens as catalysts proceeds in the following way. The ascorbic acid molecule is oxidized to dehydroascorbic acid, while the catalyst is reduced. The reduced catalyst in turn is reoxidized by atmospheric oxygen and is ready to act once more as oxidizing catalyst. The  $\text{H}_2\text{O}_2$  formed during the reoxidation of the catalyst is readily split into water and oxygen. Thus there is in the end the consumption of 1 atom of oxygen per molecule of ascorbic acid. The series of reactions may be expressed as follows:



To test the validity of this interpretation, we carried out the following experiments. To break the series of reactions in its

initial step (Equation 1) we used as inhibitor HCN, which with copper salts forms stable compounds. KCN at a concentration of 0.001 M per liter stopped entirely the oxidation of ascorbic acid by cupric chloride as catalyst at pH 7.04 (Fig. 5). To

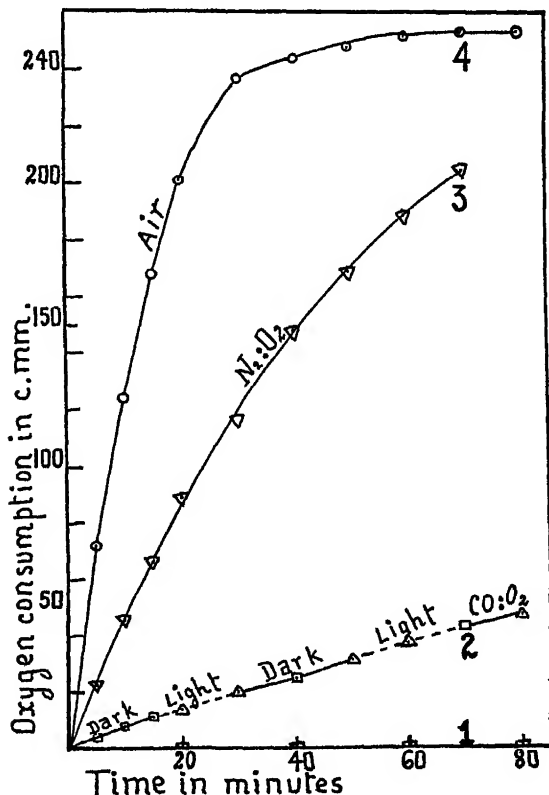


Fig. 5. The effect of oxygen concentration, HCN, and CO on the catalytic power of cupric chloride on the oxidation of ascorbic acid.  $T$ , 25°; pH, 7.04;  $N_2:O_2$  mixture = 94.91:5.09;  $CO:O_2$  = 95.13:4.87. Curve 1 represents the effect of HCN (0.001 M, KCN); Curve 2, the effect of CO; Curve 3, the oxidation in  $N_2:O_2$ ; Curve 4, the oxidation in air.

inhibit the reoxidation of cuprous ions we utilized the well known action of CO to form with cuprous chloride the reversible cuprous carbonyl chloride. (Cupric chloride does not combine with CO) In these experiments, the rate of oxidation of ascorbic acid with



cupric chloride as catalyst was measured in solutions saturated with mixtures of  $\text{CO}:\text{O}_2$  and  $\text{N}_2:\text{O}_2$ , the gas phase of our system being also made of the same mixtures. The oxygen concentration in both mixtures was about the same, namely 5 per cent. The inhibition produced by  $\text{CO}$  was 84 per cent. This inhibition is reversible, for by blowing out the  $\text{CO}$  from the Warburg vessels with a stream of air, the rate of oxidation was increased. On the other hand, illumination of the Warburg vessels with a Mazda lamp of 200 watts, placed at 8 cm. from the vessels, had no effect on the rate of oxidation (Fig. 5). The formation of  $\text{H}_2\text{O}_2$ , postulated in Equation 2, during the autoxidation of cuprous chloride was noticed as long ago as 1882 by Traube (18). The difficulty

TABLE III

*Temperature Coefficient for Oxidation of Ascorbic Acid by Atmospheric Oxygen with  $\text{Cu}^{++}$  As Catalyst*

pH 3.17; amount of ascorbic acid, 0.02 mm; amount of  $\text{CuCl}_2$ , 0.0002 mm

Experiment No.	Oxygen consumed in 30 min		$Q_{10}$
	27°	37°	
	<i>c mm.</i>	<i>c mm.</i>	
1	82.2	139.0	1.69
2	80.4	130.4	1.62
3	84.4	138.7	1.64
Average . . . . .			1.65

of recognizing its presence when such autoxidation occurs in atmospheric oxygen, due to its rapid decomposition into water and oxygen, was demonstrated by Wieland and Franke (19).

*Temperature Coefficient for Oxidation of Ascorbic Acid*

The fact that diminishing the oxygen concentration in the Warburg vessels from 21 per cent (air) to 5 per cent produced an inhibition of 56 per cent on the rate of oxidation of ascorbic acid with copper as a catalyst (Fig. 5) suggested that in the successive reactions which occur in this process the reoxidation of cuprous ions by atmospheric oxygen was performed at the slowest rate, thus determining the net tempo of the whole reaction. Filson and Walton (20) have made the statement, although no

data are found in their paper, that a change in temperature of  $10^{\circ}$  (from  $30^{\circ}$  to  $40^{\circ}$ ) "had very little effect on the rate of reaction" of cuprous to cupric chloride by atmospheric oxygen. We determined the temperature coefficient for the oxidation of ascorbic acid with copper as catalyst at  $27^{\circ}$  and  $37^{\circ}$ , at pH 3.17, and a ratio of ascorbic acid to  $\text{CuCl}_2$  of 100:1. Three experiments, with three vessels for each experiment, were performed, and a value of 1.65 for  $Q_{10}$  was found (Table III), a low value, which would seem to be in agreement with Filson and Watson's claim and would appear to favor the suggestion made above that the reoxidation of cuprous chloride is the reaction which governs the rate of oxidation of ascorbic acid.

*Reversible Reduction of Ascorbic Acid Oxidized by Atmospheric Oxygen with Copper As Catalyst*

Tillmans, Hirsch, and Dick (21) drew a clear distinction between the reversible and irreversible oxidation of ascorbic acid, maintaining that the first kind of oxidation could be brought about by iodine,  $\text{H}_2\text{O}_2$ , or Clark's 2,6-dichlorophenol, and be reversed with  $\text{H}_2\text{S}$ , whereas oxidation by atmospheric oxygen produced an irreversible oxidation. The reversible oxidation of ascorbic acid by mild oxidizing agents has been confirmed by Johnson (22). The so called irreversible oxidation by atmospheric oxygen has been accepted. The degree of reversibility of the oxidation product of ascorbic acid is, we believe, a function of the hydrogen ion concentration and is independent of the nature of the oxidizing agent. Borsook and Keighley (6) have already pointed out that the difficulty in obtaining thermodynamically reversible potentials with ascorbic acid in solutions beyond pH 6.0 is due to its irreversible oxidation, and Wurmser and de Loureiro (23) determined by the indicator technique, at different pH values, the half life of the reversible oxidation product of ascorbic acid. The experiments on the chemical reversibility of ascorbic acid oxidized by atmospheric oxygen were performed as follows: Solutions of ascorbic acid in buffers of varying pH values were divided into four parts. Part A was transferred to a Warburg vessel containing 0.2 cc of  $\text{CuCl}_2$  (0.7 mm per liter), where it was shaken at  $25^{\circ}$  until oxidation was complete; Part B, the control, was immediately titrated with 0.01 N iodine; Parts C

and D, to which  $\text{CuCl}_2$  was added (0.7 mm per liter), were shaken in air until there was no further oxygen consumption in the Warburg vessel. Part C was titrated and invariably found to contain no more ascorbic acid; Part D was kept with  $\text{H}_2\text{S}$  bubbling through it for 3 hours. (In the case of solutions of pH values of 6.6 and 7.3, some citric acid was added to bring the pH to 4.0 in order to have  $\text{H}_2\text{S}$  in its undissociated form.) The flasks were then well stoppered and kept in the ice box overnight. On the following day purified nitrogen was passed through them for 5 hours, and the solutions then filtered (to separate the copper

TABLE IV  
*Reversible Reduction of Ascorbic Acid Oxidized by Atmospheric Oxygen  
 with  $\text{CuCl}_2$  As Catalyst*  
 Concentration of  $\text{CuCl}_2$ , 0.77 mm per liter;  $T$ , 25°.

pH	Ascorbic acid		Recovered	Time shaken in air
	Before oxidation	Recovered after reduction with $\text{H}_2\text{S}$		
	mg	mg	per cent	min.
3.17	14.60	14.50	100.0	60
4.16	10.00	10.00	100.0	52
5.06	10.00	9.60	96.0	40
5.10	9.21	8.19	89.0	60
6.60	6.43	4.65	72.3	29
6.70	12.00	8.28	69.0	30
7.36	13.00	2.49	19.2	30
7.60	14.60	0.69	4.7	60

sulfide) and titrated with iodine. As can be seen in Table IV, below pH 4.16 ascorbic acid was entirely recovered after reduction of the oxidized form by  $\text{H}_2\text{S}$ . At pH 5.0 there was also almost complete recovery; at pH 6.6, 70 per cent recovery; but at pH 7.6, there was only 4 per cent recovery. We attempted to reduce the dehydroascorbic acid with hydrogen in the presence of colloidal palladium at pH values of 6.60 and 3.17. At the end of 3 hours we were unable to detect any reduction. Similar negative results have been reported by Tillmans and coworkers (21) and by Daubney and Zilva (24).

## SUMMARY

Ascorbic acid is not autoxidizable in acid and neutral solutions up to pH 7.0. It is autoxidizable in alkaline solutions, the rate of autoxidation increasing considerably as the pH increases. Among the metallic salts tested (Mn, Ni, Fe, Co, Ca, Cu) copper is the only catalyst for the oxidation of ascorbic acid, its catalytic action being noticed in concentrations as small as 46 micrograms of copper per liter. This catalytic effect is completely inhibited by 0.001 M KCN, and to the extent of 80 per cent by mixtures of CO:O<sub>2</sub> of 95:5. When this oxidation is performed in acid and neutral solutions, there is an uptake of oxygen of 1 atom per molecule of the substrate. The oxidized form can be completely reduced by H<sub>2</sub>S up to pH 5.0. Above this pH the amount of ascorbic acid recovered gradually decreases until at pH 7.6 only 4 per cent of the oxidized form can be recovered as ascorbic acid. Hydrogen in the presence of colloidal palladium does not reduce oxidized ascorbic acid.

The temperature coefficient for the oxidation of ascorbic acid by oxygen with copper as catalyst is 1.65.

Electromotively active oxidation-reduction systems of suitable potential can also act as oxidizing catalysts. The hemochromogens of nicotine, pyridine, and pilocarpine, taken as representative examples, were found to act as catalysts. The mechanism of the oxidation of ascorbic acid by atmospheric oxygen in the presence of catalysts has been discussed.

After this paper was sent in for publication there came to the authors' attention two recently published communications bearing a relation to our experiments. Mawson (25) and Kellie and Zilva (26) have reported on the stability of ascorbic acid in the absence of catalysts, and on the catalytic power of copper. Kellie and Zilva report also that "the oxidation catalysed by Cu was very much slower when NaCl (0.1 M) was present." We found no such inhibiting effect in our experiments. In fact, 0.02 mM of ascorbic acid in phosphate buffer + 0.1 M NaCl, with 0.0002 mM of CuCl<sub>2</sub> as catalyst (final pH, 6.00), was half oxidized in 16.2 minutes. In Table II it can be seen that at the same pH value, without NaCl, ascorbic acid was half oxidized in 15 minutes. On the other hand, the addition of amino acids, through the

formation of copper complex salts, retarded the rate of oxidation considerably. Thus in the presence of 0.1 M glycine (pH 6.00), half oxidation of the ascorbic acid required 30 minutes. (The copper-glycine complex gives no color reaction with Kolthoff's reagent.)

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## STUDIES ON PANCREATIC LIPASE. I

BY S. S. WEINSTEIN AND A. M. WYNNE

(From the Department of Biochemistry, University of Toronto,  
Toronto, Canada)

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The study of the hydrolytic action of pancreatic lipase demands the recognition of several factors, only by the rigorous control of which is it possible to secure experimental data which are readily reproducible. Among these factors are four which are of primary importance. They are concerned with (1) adequate control of pH, (2) proper emulsification of the digestion mixture, (3) the use of enzyme preparations free from insoluble impurities, (4) the adoption of the initial velocity of hydrolysis as the basis of comparison in kinetic investigations. In the present work an effort has been made to control experimental methods and conditions in conformity with all four factors.

The purpose of the work was twofold: (1) the preparation of active, homogeneous, and stable solutions of pancreatic lipase by a method which yields products of consistent activity and uniform nitrogen content, (2) the application of methods of measuring the initial velocity of reaction to the study of the relative rates of hydrolysis of various substrates and the influence of pH, nature of buffer, enzyme concentration, and substrate concentration on the rate of hydrolysis.

### *Materials and Methods*

✓ *Enzyme Preparation*—Fresh pig pancreas was freed of superficial fat and extracted with ether, acetone-ether, and acetone in a manner essentially similar to that described by Willstatter and Waldschmidt-Lertz (1). The fat-free product, dried and ground to a fine powder and kept in a desiccator at 5°, retained its complete lipolytic activity for more than a year. Extracts were prepared by triturating the powder (8 gm) with water or 50 per

cent aqueous glycerol (100 cc.) and shaking at 37° for 3 hours. The solutions were rendered perfectly clear by prolonged centrifuging (up to 3 hours at 1800 R.P.M.) followed by Seitz filtration. On standing at 5° the clear solutions became somewhat opalescent in 6 weeks and began to lose their lipolytic activity after this time. The nitrogen content of several clarified aqueous glycerol extracts varied from 0.19 mg. to 0.26 mg. per cc., representing approximately 0.15 per cent protein. No further attempts were made to purify the enzyme. Extracts prepared by using 10, 20, 30, and 40 per cent glycerol were less stable than 50 per cent extracts; the use of higher concentrations of glycerol yielded final Seitz filtrates which were unnecessarily viscous. ✓

✓ *Egg Albumin Solution*—2 gm. of powdered egg albumin were dissolved in excess ammonium hydroxide; the solution was freed of ammonia by aeration, reduced in volume at low temperature, filtered through cotton wool, and made up to 100 cc.

✓ *Emulsifying Agent*—A 10 per cent solution of gum acacia was filtered through cotton wool, dialyzed against distilled water for 24 hours, and diluted to 5 per cent. Neutral sodium oleate served occasionally as an alternative emulsifier.

Fresh solutions of gum acacia and of egg albumin were prepared each week.

✓ *Buffer*—Ammonia-ammonium chloride buffer was prepared by mixing 66 parts of N ammonia and 134 parts of N ammonium chloride, the mixture having a pH of 8.9 at 30°.

✓ *Substrates*—Monoacetin, diacetin, triacetin, tripropionin, tributyrin, trivalerin, tricapronin, the methyl, ethyl, and propyl esters of formic, acetic, propionic, and butyric acids were used as substrates. .

✓ *Measurement of Rate of Hydrolysis of Glycerides*—Each reaction mixture, containing the following components, was rocked mechanically in an L-tube at 37°: 30 cc. of CO<sub>2</sub>-free distilled water, 2 cc. of CaCl<sub>2</sub> solution (1 per cent), 5 cc. of egg albumin solution (2 per cent), 5 cc. of buffer solution, pH 8.9, 10 cc. of emulsified substrate (concentration of glyceride in digestion mixture equivalent to 0.166 M), 1 cc. of enzyme solution. Samples (10 cc.) were removed for titration at 5 minute intervals, accurately timed by stop-watch to the nearest second, and were quickly transferred to flasks containing 25 cc. of 95 per cent alcohol. The pipette was washed several times with the alcohol solution. After 10 minutes

the precipitate was removed by filtration and 25 cc. of the filtrate were titrated with 0.05 N NaOH, with cresolphthalein. The titration value of a control filtrate obtained from a mixture containing boiled enzyme but otherwise identical with the active digest was taken to represent the zero point. To obtain reproducible results it was necessary to delay addition of the enzyme until the other components of the digestion mixture had been thoroughly emulsified by vigorous shaking and had attained the temperature of the water bath. A small glass vessel containing the enzyme was then dropped into the tube and the latter was shaken by hand, in the bath, for 30 seconds. A progress curve was constructed for each digestion and the initial velocity of hydrolysis was evaluated. The initial rate was usually constant for 6 or 8 minutes under the conditions adopted; in cases where linearity fell off more quickly initial velocities were determined by the method of Hanes (2). The pH was measured electrometrically.

With the aid of this procedure it was possible to obtain practically identical values for the initial rate of hydrolysis of a triglyceride, present in a given concentration, by equal amounts of enzyme extracts prepared in a standardized manner from the same lot of pancreas powder at different times extending over several months. ✓

† *Measurement of Rate of Hydrolysis of Aliphatic Esters*—In the case of these compounds it was not possible to determine so accurately the initial rate of hydrolysis due to the fact that the "esterase" activity of pancreatic extracts is relatively low. The changes occurring in 30 minutes at 37° were, therefore, taken as the basis of comparison. Each reaction mixture contained the following materials: 10 cc. of CO<sub>2</sub>-free distilled water, 1 cc. of CaCl<sub>2</sub> solution (1 per cent), 1 cc. of egg albumin solution (2 per cent), 2 cc. of gum acacia solution (5 per cent), 5 cc. of buffer solution, pH 8.9, 0.085 M substrate, 2 cc. of enzyme solution. At the end of the digestion period the reaction in each tube was stopped by the addition of 30 cc. of 95 per cent alcohol and the acid in the filtered solution was measured by titration.

#### EXPERIMENTAL

✓ The data in Table I serve to illustrate the order of magnitude of the titration figures obtained, the substrate having been present initially in a concentration corresponding to 0.166 M. The figures



represent the amount of acid liberated in 7.14 cc. of a digestion mixture prepared as previously described.

*Hydrolysis of Mono-, Di-, and Triacetin*—The initial rate of liberation of acid from 1,3-diacetin was exactly twice that from monoacetin, initially present in equimolecular concentration, indicating that glyceride linkages 1 and 3 are broken at the same rate. But the rate of liberation of acid from triacetin, present in the same molar concentration, was only 2.66 times that from monoacetin, suggesting that the middle ester linkage is less readily attacked than the two end-linkages. It is assumed that the end-linkages in triacetin, as in diacetin, are hydrolyzed at the same rate. In all experiments control tubes containing no enzyme were incubated along with experimental tubes.

+ *Rates of Hydrolysis of Triglycerides of Lower Fatty Acids*—The triglycerides previously listed and present, in their respective digests, in the same concentration (equivalent to 0.166 M) were

TABLE I  
*Hydrolysis of Tributyrin at 37°*

Time, min	0	5	10	15	20	25
0.1 N acid liberated, cc	0	0.65	1.30	1.85	2.25	2.65

hydrolyzed at different initial rates. In every case tripropionin was hydrolyzed most rapidly; a typical experiment yielded the following results, the figures being related to an arbitrary value of 100 for tripropionin.

Substrate	Relative initial rate of hydrolysis
Triacetin . . . . .	53
Tripropionin . . . . .	100
Tributyrin . . . . .	87
Trivalerin . . . . .	40
Tricaproin . . . . .	71

The rates of hydrolysis of the various triglycerides do not appear<sup>1</sup> to fall in any logical order in relation to the molecular weights of the compounds.

*Hydrolysis of Esters*—In Table II are recorded titration values

which express the degree of hydrolysis of different esters, each present initially in 0.085 M concentration. The amount of hydrolysis increases with the length of the fatty acid chain but decreases with increase in the length of the alcohol chain. Glick and King (3) observed that the inhibitory effects of alcohols (methyl to nonyl) on liver esterase increase with increase in length of the carbon chain. Each titration figure in Table II represents a corrected value after taking account of the acid liberated in control tubes containing boiled enzyme. Of all the substrates in control tubes, the formates were the most unstable; the spontaneous hydrolysis of ethyl formate was the most rapid.

✓ *Enzyme Concentration and Reaction Velocity*—In a study of the hydrolysis of olive oil, during a period of 60 minutes, by different amounts of a pancreatic lipase preparation, McGillivray (4)

TABLE II  
*Hydrolysis of Related Esters*

	0.05 N acid in 25 cc of filtrate			
	Formate	Acetate	Propionate	Butyrate
	cc	cc	cc	cc
Methyl . . . . .	1.05	2.00	2.10	2.45
Ethyl . . . . .	0.30	1.80	1.90	2.00
Propyl . . . . .	0.15	1.65	1.80	1.85

obtained titration figures which, when plotted against relative enzyme concentration (abscissa), gave a parabola. In view of the fact that the linearity of progress curves representing lipolysis frequently disappears after the first few minutes of digestion, the use of any titration figures other than those representing initial velocities in establishing a relationship between enzyme concentration and reaction velocity is open to criticism. It has been found in the present work that, over a considerable range of enzyme concentration, the initial velocity is directly proportional to the concentration. ✓

+ *pH and Reaction Velocity*—In Table III are recorded pH optima obtained by several workers for pancreatic enzymes acting under various conditions. Obviously, it is impossible to designate any specific pH as the optimum for the enzyme. Since many of the

recorded data were based on titration values obtained after digestion intervals considerably beyond the time when it might be expected that the linearity of the progress curves had disappeared and that the pH had reached levels appreciably below the initial values, experiments were conducted to determine the relation between pH and initial velocity. The certainty of optimum pH values based on rates of lipolysis other than initial rates is open to serious doubt.

With phosphate-borate buffers (Kolthoff (8)) the optimum was found to be pH 7.2 for both tripropionin and methyl butyrate. The former substrate exhibited a narrow optimum pH range and the activity-pH curve dropped sharply on either side; the optimum range for methyl butyrate was much broader. In the presence of

TABLE III  
*Action of Pancreatic Enzymes under Various Conditions*

Reference	Substrate	Buffer	Optimum pH found
Umeda (5)	Olive oil	Phosphate	7.4
Willstatter and Waldschmidt- Leitz (1)	" "	NH <sub>3</sub> -NH <sub>4</sub> Cl	9.2
Platt and Dawson (6)	Tributyrin	"	8.3
	Ethyl butyrate	Phosphate	6.9
	" "	Borate	8.5
Anrep, Lush, and Palmer (7)	Olive oil	Phosphate	7.8

glycine buffer most rapid hydrolysis of tripropionin occurred at pH 9.3. In no case was there any evidence of a dip in the curve such as that observed by Sobotka and Glick (9) who studied the esterase activity of liver and pancreas.

*Substrate Concentration and Reaction Velocity*—Recent observations made by Sobotka and Glick (10) on the influence of substrate concentration on the rate of hydrolysis of various substrates by the lipolytic enzymes of liver and pancreas have led these workers to conclude that the enzyme systems concerned function in conformity with the theory of Michaelis and Menten. In each of their experiments four concentrations of substrate were usually employed, all within the limit of its solubility. In the present work ten concentrations of tripropionin, corresponding to the

range 0.016 to 0.16 M, were used in each of three experiments. The initial velocity was directly proportional to the substrate concentration up to 0.128 M, after which the curve flattened, analysis of the data did not reveal satisfactory agreement with the Michaelis theory. Further experiments on the affinity of the lipase for various substrates are now in progress.

#### SUMMARY

✓ The rate of hydrolysis of glycerides and alkyl esters of fatty acids by pancreatic lipase was investigated, with the results summarized below. The digestions were carried out in the presence of egg albumin, gum acacia, and calcium chloride.

1. Triglycerides were hydrolyzed at initial rates decreasing in the following order: tripropionin, tributyrin, tricaproin, triacetin, trivalerin.

2. The initial rates of acid liberation from equimolecular amounts of monoacetin, 1,3-diacetin, and triacetin were in the ratio 1:2:2.66. The middle ester linkage of the triglyceride is, apparently, more slowly hydrolyzed than the two end-linkages.

3. On comparing the rates of hydrolysis of the methyl, ethyl, and propyl esters of formic, acetic, propionic, and butyric acids, it was observed that the rate increased with an increasing number of carbon atoms in the acid radical but decreased with an increasing length of the alcohol radical.

4. With phosphate buffer the initial rate of hydrolysis of tripropionin and of methyl butyrate was greatest at pH 7.2. With glycine buffer the optimum pH for the hydrolysis of tripropionin was 9.3.

5. The initial velocity of hydrolysis varied directly as the enzyme concentration.

6. The initial velocity was directly proportional to the concentration of tripropionin in the range corresponding to 0.016 to 0.16 M. Velocities calculated from the Michaelis equation did not agree satisfactorily with the observed velocities.

The writers are indebted to Mr. L. Rabinowitch for permission to use the results of experiments on the influence of glycine buffers on reaction velocity.

✓

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## STUDIES ON PANCREATIC LIPASE

### II. INFLUENCE OF VARIOUS COMPOUNDS ON THE HYDROLYTIC ACTIVITY

BY S. S. WEINSTEIN AND A. M. WYNNE

(From the Department of Biochemistry, University of Toronto,  
Toronto, Canada)

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In the case of lipolytic enzymes practically nothing is known concerning the chemical nature of the groupings, in their molecules, which are associated with their peculiar activity. ✓ The precipitation experiments of Glick and King (1) suggest that either pancreatic lipase itself is a globulin or its activity is very intimately bound up with globulin. The work of Murray (2) indicates that the enzyme contains essential chemical groups which react with the carbonyl group of ketones. ✓

The experiments to be reported in the present paper are concerned with the effects of various compounds on the activity of the enzyme and were undertaken with the hope that the results might lead to some further understanding of the nature of the reactive groups and assist in the formulation of a logical interpretation of lipase action.

#### *Materials and Methods*

The substances whose effects were investigated included ketones, aldehydes, cyanides, cyanate, thiocyanate, heavy metals, thiol compounds, quinone, polyhydric alcohols, halogen derivatives of fatty acids, halides, bile salts.

The degree of inhibition or of activation was calculated after determination of the initial velocity of hydrolysis of tripropionin in the presence and absence of the various substances. The enzyme preparation was a clarified 50 per cent of glycerol extract of pig pancreas powder; the experimental procedure for determining the rate of hydrolysis was identical with that previously

described (3). The digestion mixtures were buffered at pH 8.9 with ammonia-ammonium chloride buffer; the temperature was 37°. Whenever necessary the solutions of the compounds were adjusted to the proper pH before addition to the enzyme solution. Prior to their addition to the other components of the digestion mixture the enzyme and the compound under investigation were allowed to remain in contact at 37° for varying periods of time. The enzyme to be added to control tubes was similarly treated with water equivalent in volume to that of the inhibitor solution employed. It was frequently observed that the degree of retardation or of acceleration of the activity of the lipase was dependent upon the duration of this preliminary contact.

Each reaction tube contained the following substances: 30 cc. of CO<sub>2</sub>-free distilled water, 2 cc. of CaCl<sub>2</sub> solution (1 per cent), 5 cc. of egg albumin solution (2 per cent), 5 cc. of ammonia-ammonium chloride buffer, pH 8.95, 10 cc. of tripropionin emulsion, giving a concentration in the digestion mixture equivalent to 0.166 M, 2 cc. of enzyme solution containing the compound under investigation. In the experiments on the effects of the halides, calcium chloride was omitted as a standard constituent.

It should be stated that, in considering the data in Tables I to VIII which follow, inhibitions or activations of less than 5 per cent of the normal rate are regarded as being of doubtful significance. In each table the figures represent the relative initial velocities of hydrolysis effected by the enzyme treated with the various compounds, the results being expressed as percentages of the initial rate of hydrolysis obtained with untreated enzyme.

#### EXPERIMENTAL

*Ketones and Related Compounds*—In confirmation of Murray's findings, ketones are observed (Table I) to exercise marked inhibitory effects, probably by attachment of the carbonyl group to reactive groups in the enzyme molecule. The degree of inhibition depends upon the time of preliminary contact between enzyme and inhibitor and is influenced by the molecular volume of the inhibitor, by the number of carbonyl groups present, and by the presence or absence of the benzene ring in the inhibitor molecule. Conversion of a ketone to the corresponding oxime (*cf.* ethyl methyl ketoxime) does not destroy the inhibitory capacity of the

compound. The reactive forces in the oxime are probably the residual valencies of the nitrogen and oxygen atoms. The molecular volumes listed in Tables I and II were calculated from the data of Sugden (4).

*Aldehydes*—With the exception of paraldehyde, all aldehydes investigated caused marked inhibition of activity (Table II). The degree of inhibition increased with rising molecular volume in the series comprising formaldehyde, acetaldehyde, butyraldehyde, and heptaldehyde; this progressive increase becomes appar-

TABLE I  
*Influence of Ketones on Rate of Hydrolysis*  
Concentration of inhibitors in digestion mixtures,  $3.7 \times 10^{-3}$  M.

	Time of preliminary contact of enzyme with inhibitor						Molecular volume*
	0 min	5 min	10 min	15 min	30 min	60 min.	
	Rate of hydrolysis as per cent of control rate						
Acetone .	105	75	57	48	45	37	161 5
Ethyl methyl ketone	100	100	76	47	37	33	198 2
Diethyl ketone ...	100	74	52	41	40	29	236 2
Dipropyl “	100	79	50	40	25	15	240
Methyl <i>n</i> -hexyl ketone .	53	28	25	19	14	12	260
Acetyl acetone .....	0	39	29	16	2	0	207
Acetophenone. ... ..	42	2	0	0	0	0	310
Cyclohexanone. ....	74	58	42	34	14	7	249
Cyclopentanone . . .	86	50	44	31	25	19	212 5
Ethyl methyl ketoxime ..	100	60	55	32	24	10	228 8

\* Calculated from the data of Sugden (4).

ent after 10 minutes preliminary contact with the enzyme. On the other hand paraldehyde, present in the same concentration, was very much less inhibitory, although its molecular volume is greater than that of heptaldehyde. The normally reactive carbonyl groups of the aldehydes are not free in paraldehyde, the oxygen atoms being components of the ring. Aldol, containing a secondary alcohol group in addition to the aldehyde group, was more strongly inhibitory than butyraldehyde. Benzaldehyde was very strongly inhibitory, slightly more so than heptaldehyde, although their molecular volumes are almost identical. Benzene



itself was found to be inert when present in an amount equivalent to the concentration of the other substances tested. However, benzene is not sufficiently soluble in water to yield a solution of this concentration, to this fact one might attribute its apparent inertness, or it may be due to the absence of a reactive group in the molecule. On comparing the inhibitory capacities of heptaldehyde and methyl *n*-hexyl ketone, compounds having approximately equal molecular volumes, it is apparent that the aldehyde is much more toxic than the ketone.

It should be stated that the concentrations of the various compounds listed in Tables I to VIII represent the concentrations of

TABLE II  
*Influence of Aldehydes on Rate of Hydrolysis*  
Concentration of inhibitors in digestion mixtures,  $3 \times 10^{-3}$  M.

	Time of contact						Molecular volume
	0 min	5 min	10 min	15 min	30 min	60 min	
	Rate of hydrolysis as per cent of control rate						
Formaldehyde . . . . .	111	86	75	72	61	54	82 2
Acetaldehyde . . . . .	92	84	64	50	34	24	121 2
Butyraldehyde . . . . .	73	67	47	30	20	2	198
Aldol . . . . .	65	43	36	2	0	0	208
Heptaldehyde . . . . .	43	15	2	0	0	0	267
Paraldehyde . . . . .	97	97	97	97	93	87	279
Benzaldehyde . . . . .	63	2	0	0	0	0	265

the substances in the digestion mixtures. During preliminary contact with the enzyme each compound was present in the enzyme solution in a concentration 27 times the recorded value.

*Heavy Metals*—In Table III are summarized the results of experiments on the inhibitory powers of salts of heavy metals determined in the usual way after 15 minutes contact of enzyme with metallic compound. In contrast to their relative inertness, in concentrations of the order of  $10^{-3}$  M, toward the proteinase component of similar enzyme preparations (5), the salts of heavy metals exercised a marked inhibitory influence on the activity of the lipase when present in concentrations of the order of  $10^{-5}$  M. From the point of view of absolute values the figures in Table III

are of doubtful significance, however, since the enzyme preparations, like many others which have been subjected to studies of this kind, probably contained quite large amounts of impurities. Confirming the results of Platt and Dawson (6) and of Corran (7), copper was found to be more toxic than mercury in the same concentration. The opposite effect was obtained by Jacoby (8) for urease. Ferric sulfate was more toxic than the ferrous salt.

*Cyanide, Cyanate, and Thiocyanate*—Table IV shows the results obtained with these compounds. Both KCN and  $\text{CH}_3\text{CN}$  appeared to activate the lipase, the former more extensively than the latter. In each case the range of concentration associated with activation

TABLE III  
*Influence of Salts of Heavy Metals on Rate of Hydrolysis*  
Preliminary contact of enzyme with inhibitor, 15 minutes

	Salt concentration ( $\times 10^{-5}$ M)			
	0.5	1.0	1.5	2.0
	Rate of hydrolysis as per cent of control rate			
$\text{CuSO}_4$ . . . . .	85	54	42	20
$\text{HgCl}_2$ . . . . .	100	81	72	56
$\text{Fe}_2(\text{SO}_4)_3$ . . . . .	91	78	51	45
$\text{FeSO}_4$ . . . . .	100	100	83	79
$\text{CoCl}_2$ . . . . .	77	54	50	35
Colloidal iron* . . . . .	93	93	76	73

\* Concentration expressed in terms of molarity.

was quite narrow. Cyanate and thiocyanate were inert when used in the same concentrations as the cyanides. By plotting per cent activation against KCN concentration a smooth curve was obtained, showing maximum activation with about  $6 \times 10^{-6}$  M KCN. This, of course, is the concentration in the digestion mixture; in preliminary contact with enzyme, as previously stated, the cyanide was present in 27 times this concentration. As shown in Table V it requires an appreciable time for the full influence of the cyanide on the enzyme to become manifest (in this experiment only after 15 minutes preliminary contact at  $37^\circ$ ).

Activation by cyanide has previously been observed in the case of several other enzymes including papain, bromelin, cathepsin;

the subject has been reviewed by Grassmann (9), by Murray (10), and by Tauber (11). Potato amylase was shown by Barker (12) to be susceptible to cyanide activation, and very recently Farber and Wynne (5) made a similar observation in the case of pancreatic proteinase.

TABLE IV

*Influence of Cyanide and Cyanates on Rate of Hydrolysis*

Preliminary contact with enzyme, 15 minutes

	Concentration ( $\times 10^{-6}$ M)									
	1	2.5	5	10	15	20	30	40	50	500
	Rate of hydrolysis as per cent of control rate									
Potassium cyanide ..	110	130	149	135	96	65				
Methyl cyanide (acetoni- trile) .		109	118	113	100					
Potassium cyanate. .			100	104	104	102	100	100	100	100
" thiocyanate			97.5	100	97.5	100	103	100	103	100

TABLE V

*Influence of Duration of Preliminary Contact of Enzyme with Cyanide on Degree of Activation*

Concentration of KCN in digestion mixture,  $5 \times 10^{-6}$  M.

Time of contact	Rate of hydrolysis as per cent of control rate
min.	
0	100
5	120
10	130
15	150
30	149
60	150

*Sulfur Compounds*—In recent years several enzymes have been shown to be affected by the presence of thiol compounds; in some cases activation occurs. The influence of such compounds on enzyme activity has recently been reviewed by Bersin (13). Since pancreatic lipase has not, apparently, been investigated from this point of view, the experiments summarized in Table VI were carried out.

The enzyme and sulfur compound were allowed to remain in

preliminary contact for 60 minutes; otherwise the conditions were identical with those of previous experiments. Each of the compounds, cysteine, thioglycolic acid, and sodium hydrosulfite, caused definite activation when present in certain concentrations. Sodium sulfide and sodium bisulfite were inhibitory. The effects could not be attributed to change of pH since this was carefully controlled in all experiments.

The extent of the present work does not permit any conclusion as to the mechanism of lipase activation by thiol compounds. There is, however, no evidence to indicate that they exercise their effects by the reduction of toxic quinones, present as impurities, to dihydric phenols in the manner suggested by Quastel (14) to explain the protective action of thiol compounds upon urease in

TABLE VI  
*Influence of Sulfur Compounds on Rate of Hydrolysis*  
Preliminary contact with enzyme, 60 minutes

	Concentration in digestion mixture ( $\times 10^{-4} M$ )								
	1	2	3	4	5	6	7	10	25
	Rate of hydrolysis as per cent of control rate								
Cysteine		100	112		127		124		
Thioglycolic acid . . . .	100		96		108		115		
Sodium sulfide . . . . .			100		95		89	84	
“ hydrosulfite . . . . .		100		107		116		122	
“ bisulfite					90		78	68	60

the presence of quinone or of compounds containing quinone as an impurity. It is probable that the amounts of quinone and related compounds in the enzyme preparations used in our experiments were negligible in view of the high solubility of these compounds in the solvents used in the treatment of the wet pancreas. Furthermore, quinone in fairly high concentration has been shown to be only moderately inhibitory toward pancreatic lipase, very much less so than toward urease, which Quastel found to be 98 per cent inhibited by quinone in a dilution of 1:2,500,000.

*Phenol, Polyhydric Phenols, and Related Compounds*—The effects of several phenolic compounds on the activity of the enzyme after 60 minutes preliminary contact at 37° were examined. Four concentrations of each compound in the digestion mixture were

used; namely, 5, 7, 10, and  $25 \times 10^{-4}$  M; in preliminary contact with enzyme the compounds were present in concentrations 27 times these values. Included in the experiment were phenol, *m*-cresol, *o*-cresol, nitrophenol, catechol, resorcinol, hydroquinone, orcinol, guaiacol, phloroglucinol, pyrogallol, and inositol. Of these, only nitrophenol, catechol, resorcinol, hydroquinone, and orcinol had any appreciable influence, the degrees of inhibition by the highest concentration being 16, 12, 19, 33, and 20 per cent respectively. The inhibitory capacity of the dihydric phenols appears to increase with increased separation of the hydroxy groups. The presence of the methyl group, as in the cresols and orcinol, does not affect the toxicity of the parent compound; the nitro group, however, definitely increases the toxic properties of the phenol.

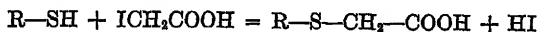
*Orthoquinone*—This compound, after 60 minutes contact with enzyme at  $37^\circ$ , caused inhibition of activity as follows, the concentrations listed being those in the digestion mixtures.

Concentration		Inhibition
$\times 10^{-4}$ M		per cent
1	(About 1:100,000)	0
2		5
3		10
4		20

It was difficult to be certain of the results when concentrations of quinone higher than  $5 \times 10^{-4}$  M were used, owing to color interference with the titration end-point. When the results with inhibition (*cf.* Table I) by a substance such as acetophenone, in which the keto group is a part of the aliphatic chain, are compared, it is apparent that inclusion of the group in the aromatic ring reduces its effectiveness as an inhibitor.

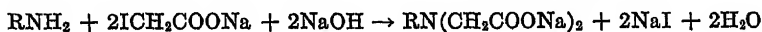
*Halogen Derivatives of Fatty Acids*—The three compounds, chloro-, bromo-, and iodoacetic acid, inhibited the activity to an extent increasing in the order named (Table VII). Dickens (15) and Michaelis and Schubert (16) have suggested possible explanations of the mechanism of enzyme inhibition by these compounds. Dickens' study of the speed of the reaction between glutathione and the three monohalogen derivatives of acetic acid

led him to conclude that the reaction is bimolecular and results in the liberation of the corresponding halide and the formation of a thio ether



Extending the consideration of this reaction to the inhibition of glyoxalase by monoiodoacetate, Dickens concluded that the inhibitor inactivated the enzyme system by combination with the coenzyme, glutathione. He found, furthermore, that iodo-, bromo-, and chloroacetic acids reacted with glutathione with relative speeds expressed by the ratio 15:9:0.15. In the present work it was found that, when present in the concentration  $10^{-4}$  M, the three compounds inhibited lipase activity to an extent expressed relatively by the figures 16, 11, and 4 for the iodo, bromo, and chloro acids respectively. There is, therefore, some resemblance between the relative inhibitory capacities of the compounds on the one hand and the relative speeds of their reaction with glutathione on the other. Since it has been shown that sulfhydryl compounds activate the lipase, it is quite possible the halogen acetic acids may, at least partially, inactivate the enzyme by reacting with a natural sulfhydryl activator in the manner suggested by Dickens or with an essential —SH component of the enzyme molecule.

Michaelis and Schubert have, however, pointed out that iodoacetic acid reacts quite easily with amino groups as follows:



and that the iodo acid is probably more reactive than the bromo and chloro compounds. In the case of the inhibition of enzymes by iodoacetic acid, Michaelis and Schubert have suggested that at pH 7 to 8 the point of attack of this acid may be considered as a —SH group provided this is confirmed by other evidence, but, "If the effect of iodoacetic acid occurs only at pH > 7 or 8, the point of attack may be just as well an amino group."

From the evidence available it is impossible to decide whether reaction with the —SH group or the —NH<sub>2</sub> group or with both groups takes place during the inhibition of lipase activity by the halogen acetic acids. The susceptibility of the enzyme to inhibition by aldehydes and heavy metals suggests that an amino group

may be an essential constituent of the enzyme; if so, the halogen acetic acids may quite possibly cause inactivation by union with this group.

*Halides*—Inhibition by these compounds increased in the order chloride, bromide, iodide, fluoride (Table VIII). With the exception of fluoride these anions exercised effects in conformity with their positions in the lyotropic series; fluoride, however, was quite definitely the most toxic of all. Haldane's suggestion (17) that

TABLE VII

*Influence of Monohalogen Derivatives of Acetic Acid on Rate of Hydrolysis*  
Preliminary contact with enzyme, 60 minutes.

	Concentration in digestion mixture ( $\times 10^{-3}$ M)					
	1	25	3	5	8	10
	Rate of hydrolysis as per cent of control rate					
Chloroacetic acid . . .	96			92	92	92
Bromoacetic " . . .			93		82	78
Iodoacetic " . . .		100		79	72	68

TABLE VIII

*Influence of Halides on Rate of Hydrolysis*  
Preliminary contact with enzyme, 60 minutes.

	Concentration in digestion mixture ( $\times 10^{-4}$ M)			
	5	7	10	25
	Rate of hydrolysis as per cent of control rate			
NaCl . . . . .	96	89	81	73
NaBr . . . . .	84	77	65	58
NaI . . . . .	82	74	64	52
NaF . . . . .	65	54	46	35

the inhibition of lipases by fluoride, observed by Rona and Pavlović (18), is due to its position in the Hofmeister series cannot, therefore, be regarded as offering a completely satisfactory explanation.

*Bile Salts*—Willstatter and his collaborators (19, 20) observed that bile salts activated pancreatic lipase in an alkaline medium but caused inhibition in an acid medium. Gluck and King (21) studied the effect of bile salts on the rate of hydrolysis of tributyrin

by pancreatic lipase in a medium whose pH soon fell below 7.0 as the digestion proceeded. Both sodium taurocholate and glycocholate caused inhibition. In the presence of sodium oleate the rate of hydrolysis of emulsified olive oil by lipase was accelerated by both bile salts; the pH of the mixtures was not stated. The present experiments were carried out at pH 7.2 controlled by phosphate buffer; only initial velocities were used in calculating the relative rates. The tripropionin was emulsified by means of sodium oleate and the bile salts were in preliminary contact with the enzyme for 60 minutes at 37°. At approximate neutrality the bile salts, when present in the digestion mixtures in concentrations ranging from 0.5 to  $4 \times 10^{-4}$  M, neither activated nor inhibited the enzyme.

#### DISCUSSION

✓The results as a whole show that the activity of the enzyme was affected by the presence of widely different chemical compounds. The ketones and aldehydes were definitely inhibitory when their reactive groups were free, indicating that the carbonyl group forms some sort of attachment with reactive groups in the enzyme molecule; the inhibitory capacities of closely related ketones and of closely related aldehydes bore a more or less direct relationship to the molecular volumes of the compounds. The evidence indicates that the inhibitory power of aldehydes is greater than that of related ketones having the same molecular volume. Heavy metals were inhibitory; cyanide activated the enzyme. The mechanism of this activation is not clear but preliminary experiments indicate that it is not entirely due to removal of toxic metals. Sulfhydryl compounds caused activation but apparently not, as in the case of urease, by reducing toxic quinones present as impurity to the corresponding alcohols. Phenol and the cresols were relatively inert, but the presence of the nitro group increased the toxicity. The inhibitory capacity of the three dihydroxyphenols increased with increased separation of the hydroxyl groups. Guaiacol, in which one of the —OH groups of catechol has been replaced by a —OCH<sub>3</sub> group was non-inhibitory, whereas catechol in the same concentration caused retardation. Neither of the two trihydroxy phenols investigated was inhibitory. Thus, of the hydroxy phenols, only the dihydroxy compounds were reactive.



Monohalogen derivatives of acetic acid caused inhibition in the order  $I > Br > Cl$ . The significance of this observation has already been discussed in relation to the possible mechanism of the inhibition. The halogen anions were inhibitory in the order  $F > I > Br > Cl$ . Bile salts present in digestion mixtures buffered at approximate neutrality were without appreciable influence on the activity of the enzyme.

## SUMMARY

The influence of various chemical compounds on the initial rate of hydrolysis of tripropionin by pancreatic lipase has been investigated. The compounds included ketones, aldehydes, salts of heavy metals, cyanides, cyanate, thiocyanate, thiol compounds, quinone, mono-, di-, and trihydric phenols, halogen derivatives of acetic acid, halides, and bile salts. ✓

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## THE CONSTITUTION OF THE PROSTHETIC GROUP OF CATALASE\*

BY KURT G STERN†

*(From the Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, England)*

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The constitution of the enzyme catalase is similar to that of hemoglobin. The prosthetic group is a hematin (1) and combines with a specific protein to form the enzyme molecule. Spectroscopic evidence suggests a close relationship between the porphyrin-iron complex contained in the enzyme and protohematin, the prosthetic group of hemoglobin (1). In the enzyme, however, the iron is held in the trivalent state and is unusually resistant to reducing agents (1, 2). The hematin grouping in catalase from widely different sources exhibits identical spectroscopic features (3). An apparent discrepancy in the ( $k/\text{Fe}_P$ ) ratio (where  $k$  = catalytic activity, expressed by the monomolecular reaction constant, and  $\text{Fe}_P$  = concentration of porphyrin-bound iron) between enzyme preparations of different origins still remains to be explained.

Though the dimensions of the enzyme molecule, as judged by the rate of diffusion, are probably of the same order of magnitude as those of hemoglobin (4), the protein of the enzyme complex differs from globin in the position of the isoelectric point (5) and also in its behavior towards certain precipitating agents (chloroform-alcohol). A previous synthetic approach to the problem of the constitution of the enzyme has not yielded the desired results; neither the combination of protohematin with simple nitrogen bases and various native and denatured proteins, nor synthetic

\* A preliminary note dealing with some results of this investigation has appeared (*Nature*, 136, 302 (1935))

† Now at the Department of Physiological Chemistry, Yale University, New Haven.

"methemoglobins" containing various hemins and native globin, showed a catalytic activity of a different order of magnitude when compared with the free hemins, although in some instances a definite activation was observed (6).

The present study was undertaken with the object of elucidating the precise structure of the hematin contained in the enzyme. Since spectroscopic observations are not conclusive in this field, because many isomeric porphyrin derivatives exhibit identical absorption spectra, only the actual isolation of the prosthetic group and its conversion into a derivative of characteristic properties were considered to be adequate proof. It was therefore necessary to develop a method for the preparation of highly concentrated enzyme solutions, to separate the prosthetic group from the protein component; to degrade the pure hemin to the corresponding mesoporphyrin; to prepare the dimethyl ester of the latter derivative; and to compare its melting point with that of other mesoporphyrin esters, especially with mesoporphyrin IX dimethyl ester. Furthermore, a conversion of the enzyme hemin into the corresponding hemoglobin and the comparison of the latter with the blood hemoglobin of the same species seemed to be desirable.

#### EXPERIMENTAL

##### *Development of Method of Concentrating and Purifying Catalase*

Enzyme solutions prepared from horse liver according to Zeile and Hellstrom (1) with alumina gel as adsorbent were found to have a catalytic activity of  $k = 1600$  to  $7100$ , Kat.  $f^1 = 1500$  to  $9200$  and a total iron content of  $25$  to  $43$  micrograms per cc. The large variations found are due to the differing enzyme contents of the individual horse livers used, to the efficiency of the adsorption procedure, and to the volume of elution agent used. The solutions are of brown color and will distinctly show the absorption spectrum of the enzyme in layers of thickness varying from  $1$  to  $4$  cm, depending on their catalytic activity. Table I contains the data for six different preparations.

Attempts to extract the enzyme hemin from acidified solutions of catalase with an ether-alcohol mixture met with little success. It was found necessary to concentrate the solutions further before the cleavage of the enzyme was carried out. Of the various

<sup>1</sup> Kat.  $f = k/(\text{gm. of enzyme in } 50 \text{ cc.})$ .

methods tried, ammonium sulfate fractionation, fractional freezing out, centrifuging while the frozen preparation was thawing (8), and acetone-CO<sub>2</sub> treatment, only the latter technique proved to be entirely satisfactory. A similar method was first used by Warburg and Christian (9) as one step in the preparation of the yellow oxidation enzyme from brewers' yeast.

250 cc. of a catalase solution prepared according to Zeile and Hellström were cooled in ice and saturated with carbon dioxide. On addition of 350 cc of ice-cold acetone a precipitate formed. If necessary the use of carbon dioxide in this procedure may be dispensed with, though this gas seems to promote the formation of a

TABLE I  
*Data for Catalase Preparations*

Preparation No	Liver used	Eluate obtained	k	Total solids per cc	Ash per cc	Kat f*	Fe per cc †
	<i>gm</i>	<i>cc</i>		<i>gm</i>	<i>gm</i>		<i>micrograms</i>
15	1760	850	1610	0 0205	0 0123	1567	
17	2100	700	2092	0 0160	0 0075	2615	42 6
18	1800	500	3040	0 0128	0 0080	4750	40 0
19	1800	250	4398	0 0117	0 0041	7580	42 5
28	1800	300	3410				
29	4500	500	7125	0 0155	0 0065	9200	24 8

\* Kat  $f = k/(\text{gm of enzyme in 50 cc})$ .

† The iron determinations were carried out according to Lachs and Friedenthal (7) with the substitution of a photoelectric colorimeter for the subjective comparison of the unknown with a series of standard solutions.

more readily filtrable precipitate. After shaking for half a minute, the mixture was cooled to  $-5^{\circ}$  in a refrigerator. A funnel with a pleated filter was also cooled to  $-5^{\circ}$ . The filter was then moistened with acetone and the precipitate filtered off at  $-5^{\circ}$ . The filtrate was almost colorless. The brown residue on the filter contained all of the enzyme and most of the solid matter of the enzyme preparation. The filter was removed from the cold room and the precipitate was allowed to liquefy at room temperature. The filtrate was collected and its volume was brought to approximately 10 cc by triturating the residue on the filter with a few cc of water. Filtration yields better results than centrifugation, unless the latter can be carried out near the freezing point.

The concentrates show a black-brown color and a high catalytic activity. The fact that 300 cc. of an enzyme solution of  $k = 3410$  yielded 10 cc. of a concentrate of  $k = 26,050$ , illustrates the efficiency of the method. The concentrates were almost opaque to visible light in layers of 1 cm. They showed a distinct enzyme spectrum in layers only 1 mm thick. Their light absorption between 650 and 450  $m\mu$  was solely due to the enzyme complex. However, these concentrates contained impurities, namely biliverdin, hepatoflavin, proteins, and electrolytes. Fortunately, biliverdin absorbs light only in the far red region (near 700  $m\mu$ ) and the  $\alpha$  band of hepatoflavin is situated near 440  $m\mu$ . In other respects also these pigments did not seriously interfere with the study and the isolation of the enzyme hemin.

It should be emphasized that no oxyhemoglobin, methemoglobin, or free hematin was present in the enzyme concentrates. Control experiments showed that all of the blood pigment present in the crude liver extracts was quantitatively removed at the stage of the chloroform-alcohol treatment devised for this purpose by Zeile and Hellstrom (1). There is no possibility therefore that the hemin isolated from the concentrates, instead of being part of the enzyme, was derived from contamination with blood pigment.

#### *Isolation of Prosthetic Group of the Enzyme*

The acetone- $\text{CO}_2$  treatment effected the removal of sufficient water from the enzyme solutions at low temperature to permit the cleavage of catalase by the acid-acetone method as applied to the study of respiratory heme pigments (hemoglobin (10); chlorocruorn (11)). The acetone concentrate was added drop by drop to an excess of acetone containing hydrochloric acid. For 10 cc. of the concentrate at least 500 cc. of acetone containing 5 cc. of  $\text{N HCl}$  are necessary. The colorless protein precipitate settled quickly at room temperature. The supernatant gray-blue solution was filtered off and subjected to vacuum distillation below  $30^\circ$ . When all of the acetone was removed, a red microcrystalline precipitate formed in the dark green-blue<sup>2</sup> aqueous solution. The suspension was filtered by suction while still warm. The

<sup>2</sup> The pigment responsible for this color was identified as biliverdin with the kind help of Dr. R. Lemberg.

crude hemin was washed on the filter (1 cm. in diameter), resting on a Willstatter knob, with warm dilute HCl and water. The filter was dried *in vacuo* over  $\text{H}_2\text{SO}_4$ . 1800 gm of horse liver yielded from 3 to 6 mg of crude enzyme hemin. By working up 50 pounds of liver in nine portions, approximately 40 mg. were obtained. The crude hemin was dissolved in boiling propionic acid. Hot 0.5 per cent HCl (half the volume of the propionic acid) was added. On slow cooling to room temperature, crystals of pure catalase hemin formed. Another crop of crystals was obtained by adding more HCl to the warmed mother liquor. 9 mg. of black-violet hemin crystals were obtained in this manner. The crystals were washed in the centrifuge with dilute HCl and water and dried over  $\text{P}_2\text{O}_5$  *in vacuo*.

#### *Constitution of Hemin Group of Catalase*

*Conversion into Mesoporphyrin Ester*—In order to determine the configuration of the side chains in the catalase porphyrin, the hemin was deprived of its iron and reduced to the mesoporphyrin stage. 9 mg. of the recrystallized enzyme hemin were suspended in 2 cc. of a mixture of 1 part hydriodic acid (sp. gr. 1.94) and 7.5 parts of glacial acetic acid. The suspension was boiled for 1 minute. After cooling, the deep red solution was poured into 10 cc. of a solution containing sodium sulfite and sodium acetate (The addition of sulfite, which serves to remove the excess of iodine, was suggested by Dr. R. Lemberg.) The porphyrin formed by the reaction was extracted by ether. The mesoporphyrin fraction was extracted from the ether solution by 20 cc. of 2 per cent HCl, and again driven into ether by addition of sodium acetate. The ether solution was evaporated under diminished pressure at low temperature. The resulting crystalline mesoporphyrin was treated for 20 hours at room temperature with 10 cc of dry methyl alcohol saturated with dry HCl. The methyl alcohol and HCl were removed *in vacuo* and the residue was dissolved in a small amount of chloroform. The solution was diluted with ether. The ester was extracted with 5 per cent HCl and transferred into fresh ether after addition of sodium acetate. The red-violet ether solution was twice washed with water and evaporated in a stream of dry nitrogen on a water bath at 30°. The residue was dried *in vacuo* over  $\text{H}_2\text{SO}_4$ . A further purification

was effected by recrystallization from pyridine-methyl alcohol. The first crop of crystals weighed 1 mg. By treating the mother liquor with more methyl alcohol, another small batch of crystals was obtained.

The spectrum of the isolated mesoporphyrin was found to be identical with that of a sample of mesoporphyrin prepared from blood hemin and also with that of a specimen of pure mesoporphyrin IX. (The writer is indebted to Professor O. Warburg and Dr. E. Negelein for this sample.) The absorption bands were measured with a Hilger wave-length spectrometer. The spectra of the solutions to be compared were always projected simultaneously by means of a comparison prism. The following values were obtained with ether containing a small amount of acetic acid as a solvent.

Absorption band No	I $m\mu$	II $m\mu$	III $m\mu$	IV $m\mu$	
Range . . . .	626 0-622 2	569 9-567 5	531 5.	527 7-524 5	504 0-482 5
Center. ....	624 1	568 7		526 1	498 2

There was no transmission beyond 427.5 *mμ*. The order of intensity of the bands was IV, I, III, II.

The spectrum of the mesoporphyrin dimethyl ester, when dissolved in chloroform containing pyridine, was found to be identical with that of a sample of pure synthetic mesoporphyrin IX dimethyl ester, which was kindly furnished by Professor H. Fischer. The readings were

Absorption band No	I <i>mμ</i>	II <i>mμ</i>	III <i>mμ</i>	IV <i>mμ</i>
Range . . .	623 5-616 5	567 5-564 5	538 5-527 5	511 0-498 2
Center . . .	620 0	566 0	533 0	504 6

The order of intensity of bands was IV, III, I, II.

Since the absorption spectra of porphyrins cannot provide definite proof for their identity, melting point determinations were resorted to. The mesoporphyrin esters are especially suitable for this purpose, because they have well defined melting points which differ with isomer forms and which will show appreciable depressions when mixtures of isomers are tested. The results in this case were: (a) mesoporphyrin dimethyl ester of the enzyme hemin, m.p. 214°; (b) synthetic mesoporphyrin IX dimethyl ester, m.p. 212-213°; (c) a mixture of approximately equal amounts of (a) and (b), m.p. 213°.

It follows that the ester prepared from the enzyme is identical with mesoporphyrin IX dimethyl ester. This means that the catalase hemin is a derivative of etioporphyrin III and that the side chains are arranged in the pattern (IX). The experimental procedure followed here is analogous to that used to prove the constitution of the mesoporphyrin derived from cytochrome *c* (12).

*Conversion into Hemoglobin*—It still remained to establish the identity of the catalase hemin with protohemin, since hemato-hemin and mesohemin will yield the same mesoporphyrin as protohemin. This could best be done by combining the enzyme hemin with native horse globin and by comparing the product obtained with horse hemoglobin.

0.9 mg. of twice recrystallized catalase hemin was dissolved in 0.5 cc. of 1 per cent sodium carbonate solution. A residue was removed by centrifuging. The deep red solution was mixed with 5 cc. of 0.49 per cent horse globin solution. In the preparation of the globin the directions given by Wu (13) for ox globin were followed. Instead of concentrating the dilute globin solution *in vacuo* over  $H_2SO_4$ , some of the water was removed by fractional freezing at  $-5^\circ$ . The clear, pale yellow solution contained 0.69 mg. of N per cc. (micro-Kjeldahl).

The alkaline methemoglobin formed was compared with horse methemoglobin resynthesized from pure horse blood hemin and the same globin solution. The absorption spectra of the two compounds were identical. The hemoglobin formed by reduction of the methemoglobin, containing the enzyme heme, with sodium hydrosulfite showed a somewhat better defined absorption band than the blood hemoglobin control. The position of the center of the band was the same in both solutions. The oxyhemoglobins formed by aerating the hemoglobin solutions showed an identical spectrum when compared in the Hilger instrument: Band I, 583.5 to 572.0; Band II, 553.0 to 529.1  $m\mu$ .

The hemin derived from catalase appears therefore to be protohemin and hence identical with the prosthetic group of hemoglobin.

#### DISCUSSION

The following biocatalysts have been found to contain hematin groupings: the oxygen-transferring (respiratory) enzyme, cyto-



chrome, catalase, and peroxidase. All of these catalysts are modeled according to the hemoglobin pattern. Although it has been demonstrated (Warburg, Kuhn, Langenbeck, Stern) that mere variation in the nitrogenous base or protein, which is attached to a given hematin residue, may alter the catalytic activity and the specificity of the resulting product, in the biogenesis of natural heme catalysts both factors—the constitution of the heme *and* of the protein component—have been varied. Peroxidase may be a possible exception (see below).

The prosthetic group of the respiratory enzyme is to be classified with the green-red hemins, other representatives of which are spirographis hemin and pheohemin *b* (14). Cytochrome *c* contains a modified protoporphyrin in which a tertiary nitrogen base has been added to the unsaturated side chains (15). Nothing definite is known concerning the hematin in peroxidase, since the enzyme preparations obtained to date have not been concentrated enough to show a selective absorption in the visible range. But since a hemochromogen spectrum identical with that from blood hemin has been obtained from peroxidase solutions (16), it is possible that the peroxidase hematin is identical with the prosthetic group of catalase which has been demonstrated by the present investigation to be protohematin. Probably all of these hemin catalysts are derived originally from etioporphyrin III, the mother substance of hemoglobin and of chlorophyll.

Much less is known with respect to the protein component of these heme catalysts, but their general properties (*e.g.* solubility and stability) suggest that each catalyst contains a different protein. Only in the case of catalase (4, 5) and recently also of cytochrome *c* (17), a few fundamental properties (isoelectric point, molecular weight) relating to the protein part of the molecule have been determined. The comparison of methemoglobin, catalase, and possibly peroxidase, in which the only difference appears to be in the nature of the protein component, demonstrates clearly the importance of specific proteins in the problem of the heme catalysts participating in cell respiration. As has been shown in the experimental part of the present paper, the enzyme catalase may be transformed into the respiratory pigment hemoglobin by exchanging the protein component.

## SUMMARY

1. The prosthetic group of catalase has been isolated in pure and crystalline form.

2. The identity of the prosthetic group with the protohematin of hemoglobin has been established by (a) the preparation from the enzyme hemin of a mesoporphyrin dimethyl ester which was shown to have the configuration (IX), and (b) by conversion of the enzyme into the hemoglobin of the same species by substituting native globin for the enzyme protein

The writer wishes to thank Professor E. C. Dodds for providing working facilities at the Courtauld Institute of Biochemistry, where the experimental part of the paper was completed.

Acknowledgment is made by the author to his wife for assistance in the preparation of the enzyme solutions.

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# THE DETERMINATION OF THIOL AND DISULFIDE COMPOUNDS, WITH SPECIAL REFERENCE TO CYSTEINE AND CYSTINE

## IV. A PRECISION METHOD FOR THE DETERMINATION OF THIOL COMPOUNDS APPLIED TO THE STANDARDIZATION OF CYSTEINE HYDROCHLORIDE\*

By KAMENOSUKE SHINOHARA†

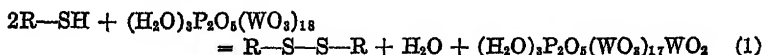
(From the Lankenau Hospital Research Institute, Philadelphia)

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Determinations of cysteine and cystine or other thiol and disulfide compounds in biological materials have so far been carried out by titration methods with iodine (1) or ferricyanide (2), or by colorimetric methods like Sullivan's naphthoquinone sulfonate method (3), Folin and Looney's phospho-18-tungstic acid method (4), etc. The present paper deals with a new method for the standardization of cysteine preparations to be used as standards for biochemical determination of cysteine and cystine.

### *Theoretical Consideration*

In Paper I of this series (5) it was pointed out that cysteine reacts under the conditions described with phospho-18-tungstic acid reagent in accordance with Equation 1



and that the intensity of the blue color produced is proportional to the concentration of the cysteine, provided the reagent is present in excess. The relation can, therefore, be expressed by Equation 2

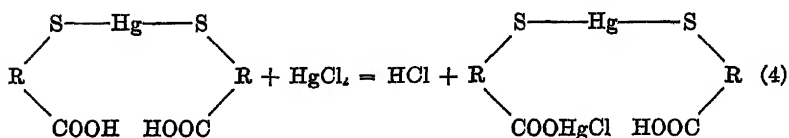
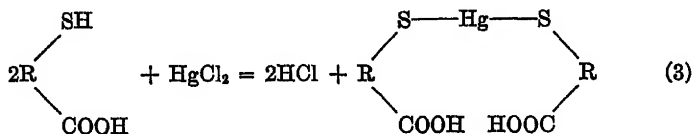
$$I_x/I_0 = C_x/C_0 \quad (2)$$

\* A part of this paper was presented before the Twenty-ninth annual meeting of the American Society of Biological Chemists at Detroit, April 10-13, 1935

† Robert McNeil Fellow of the Lankenau Hospital Research Institute.

where  $I_0$  is the color intensity of an arbitrary color standard which contains  $C_0$  ( $4 \times 10^{-4}$  M) moles per liter of cysteine, and  $I_x$  is that of a test solution containing  $C_x$  moles per liter of cysteine.

In Paper III (6) it was pointed out that cysteine reacts with  $\text{HgCl}_2$  in an acetate buffer of pH about 5 according to Equations 3 and 4



where the reaction according to Equation 3 is very rapid and proceeds to practically 100 per cent completion, while the reaction according to Equation 4 is very slow and does not take place until that of Equation 3 is completed. Equation 4 has nothing to do with the color production.

Therefore, if the phospho-18-tungstic acid reagent is added to the reaction mixture containing cysteine and  $\text{HgCl}_2$  in addition to the acetate buffer, color will be produced only by the cysteine remaining in excess, and the concentration ( $C_x$ ) of the remaining cysteine can be expressed as follows:

$$C_x = C_a - 2C_{\text{Hg}} \quad (5)$$

where  $C_a$  represents the molar cysteine concentration actually present before the addition of  $\text{HgCl}_2$  and  $C_{\text{Hg}}$  the molar concentration of  $\text{HgCl}_2$  added.

If the cysteine preparation is 100 per cent pure,  $C_a$  is expressed by Equation 6

$$C_a = \frac{W}{M} \frac{100}{V_1} \frac{V_2}{V_3} \quad (6)$$

where  $W$  is the weight (gm.) of the preparation dissolved in 0.2 M HCl (or  $\text{H}_2\text{SO}_4$ ) to make  $V_1$  cc. of stock solution,  $V_2$  cc. of which is added to the reaction mixture, which is finally made to  $V_3$  cc.

$M$  is the molecular weight of the cysteine preparation (157.62 for cysteine hydrochloride).

However, cysteine preparations are always more or less impure. Therefore, let  $C'_a$  represent this hypothetical cysteine concentration; that is, the concentration calculated on the assumption that the preparation is 100 per cent pure. Then the relation between the hypothetical ( $C'_a$ ) and the actual concentration ( $C_a$ ) is expressed by Equation 7

$$C_a = \alpha C'_a \quad (7)$$

where  $\alpha$  is the relative purity of the preparation.

When the same cysteine stock solution is used also in the color standard, the same can be said for the standard; namely,

$$C_0 = \alpha C'_0 \quad (8)$$

where  $C'_0$  represents the hypothetical cysteine concentration of the color standard, while  $C_0$  is its actual concentration.

Substituting Equations 5, 7, and 8 in Equation 2, we obtain

$$I_x/I_0 = (C'_a/C'_0) - (2/\alpha C'_0)C_{Hg} \quad (9)$$

The equation evidently does not hold beyond the intercept of the line with the  $C_{Hg}$  axis.<sup>1</sup>

Theoretically, the numerical value of the relative purity ( $\alpha$ ) can easily be calculated by introducing a value of  $I_x/I_0$  experimentally determined, together with those of  $C_{Hg}$ ,  $C'_a$ , and  $C'_0$ .<sup>2</sup> In actual determinations, however, it is preferable to employ a series of values obtained by using a series of test solutions each containing a definite amount of the cysteine preparation to be tested and a varying amount of  $HgCl_2$ . The most probable values

<sup>1</sup> Interpretation of the equation is omitted

<sup>2</sup>  $C'_a/C'_0$  is equal to the ratio of the volumes of the cysteine solution added to the test and standard solutions respectively, which is a known quantity. The ratio of the color intensity of a test solution which contains no  $HgCl_2$  to that of the color standard has been experimentally found to be equal to  $C'_0/C'_a$ . Therefore, Equation 11 can be simplified as follows with slight sacrifice in precision

$$\frac{2}{\alpha C'_0} = ((C'_a/C'_0) \Sigma x - \Sigma xy / \Sigma x^2) \quad (11-a)$$

of  $\alpha$  will then be calculated by the method of least squares, those results preferably being discarded whose intensities are less than 0.1; namely,

$$\frac{C'_a}{C'_0} = \frac{\Sigma y \Sigma x^2 - \Sigma(xy) \Sigma x}{n \Sigma x^2 - (\Sigma x)^2} \quad (10)$$

$$\frac{2}{\alpha C'_0} = \frac{\Sigma x \Sigma y - n \Sigma(xy)}{n \Sigma x^2 - (\Sigma x)^2} \quad (11)$$

where  $x$  and  $y$  represent respectively  $C_{\text{Hg}}$  and  $I_x/I_0$  of Equation 9 and  $n$  the number of solutions, the results of which have been employed in the calculation.  $\Sigma x$ ,  $\Sigma y$ , and  $\Sigma xy$  have the following significance: if  $x_1, x_2, x_3 \dots x_n$   $\text{HgCl}_2$  solutions are added to solutions containing  $C'_0$  (hypothetical) cysteine and respectively  $y_1, y_2, y_3 \dots y_n$  color intensities are obtained, then

$$\Sigma x = x_1 + x_2 + x_3 \dots + x_n \quad (12)$$

$$\Sigma y = y_1 + y_2 + y_3 \dots + y_n \quad (13)$$

$$\Sigma xy = x_1 y_1 + x_2 y_2 + x_3 y_3 \dots + x_n y_n \quad (14)$$

$$\Sigma x^2 = x_1^2 + x_2^2 + x_3^2 \dots + x_n^2 \quad (15)$$

$$(\Sigma x)^2 = (x_1 + x_2 + x_3 \dots + x_n)^2 \quad (16)$$

The relative purity can also be found by graphically interpolating or by actually determining the minimum amount of  $\text{HgCl}_2$  which totally inhibits color development. The results concerning these aspects are shown in the experimental part.

#### EXPERIMENTAL

##### *Reagents*

0.01 M  $\text{HgCl}_2$  solution. This was prepared from Merck's reagent grade preparation. Its molarity was checked by gravimetric analysis in which Hg was determined as  $\text{HgS}$ . The molarity calculated from the amount weighed and that calculated from the amount of  $\text{HgS}$  agreed within  $\pm 0.05$  per cent.

Cysteine hydrochloride. Eastman Kodak Company's and Pfanstiehl Chemical Company's preparations were used. The purity of these preparations will be described below. Other reagents are the same as described in Paper I (5) of this series unless otherwise mentioned.

The color standard is the same as described in Paper I ( $4 \times 10^{-4}$  M cysteine)

*Technique*—In each series of experiments, definite amounts of a cysteine solution are introduced into several 50 cc. volumetric flasks, each containing 10 cc. of 2 M sodium acetate, 3 cc. of 2 M acetic acid, and an amount of 0.2 M NaOH equal to the added cysteine solution. This is followed by varying amounts of 0.1 M  $\text{HgCl}_2$  solution. The cysteine solution was made by dissolving 0.3491 gm. of a commercial cysteine hydrochloride in 25 cc. of 2 M HCl and diluting it to exactly 250 cc.<sup>3</sup>

After the contents were mixed and the solutions set aside for about 1 minute, 4 cc. of phospho-18-tungstic acid reagent were added, the total volume of the contents then being made up to exactly 50 cc. by adding water. The blue-colored solutions were finally subjected to colorimetry in 5 to 10 minutes against a color standard, the composition of which is mentioned in Table I.

The values of  $\alpha$  were calculated by Equations 9, 11, and 11-a, by graphical interpolation, and by actual determination of the minimum amount of  $\text{HgCl}_2$  that totally inhibits color development.

*Relation of Color Intensity, Cysteine Hydrochloride, and  $\text{HgCl}_2$  Concentrations*—Four series of determinations, with different amounts of a cysteine solution which had been made from the same cysteine hydrochloride preparation, were carried out, their results being shown in Table I, a. When the values of  $\alpha$  of each of the series are calculated from Equation 9 by substituting separately each experimental value, their equality<sup>4</sup> within experimental errors indicates that a straight line is formed when the intensity is plotted against the  $\text{HgCl}_2$  concentrations. The straight lines made by the four series of experiments are parallel to each other.

The color intensities of the solutions containing no  $\text{HgCl}_2$  (values of  $C'_a/C'_0$ ) are seen to be in the expected ratios. 0 intensity is given by solutions of which the  $\text{HgCl}_2$  concentrations are

<sup>3</sup> This solution, therefore, corresponds to 0.01 M cysteine if the cysteine preparation is 100 per cent pure. Generally the concentration is 0.01  $\alpha$  M where  $\alpha$  is the relative purity of the preparation with respect to cysteine.

<sup>4</sup> The somewhat large deviation with 0.5 cc. of the cysteine solution is, no doubt, due to the larger errors occurring in the measurements of such small volumes. For routine procedure the use of 2 to 5 cc. of 0.01 M cysteine solution seems best.



beyond those of the intercepts of the lines with the  $\text{HgCl}_2$  concentration axis

The results of three series of experiments, obtained by using the same amount of cysteine solutions containing the same amount of different cysteine hydrochloride preparations, are shown in Table I, b. They clearly show that different preparations give different slopes.

The experimental results are seen to fulfil the propositions offered by Equation 9, and, therefore, the method serves to determine the value of  $\alpha$ , relative purity.

*Thioglycolic Acid*—Thioglycolic acid was tested in exactly the same way as cysteine, the reaction mixture having the same composition except that thioglycolic acid was substituted for cysteine. The results presented in Table II clearly show that it behaves as cysteine does.

*Reaction of Mercuric Chloride with Cystine*—The reaction was studied by Andrews and Wyman (7) and later by Simonsen (8) who showed that a precipitate of a mercury compound of cysteine is formed in the mixture. The same reaction takes place under the conditions of the present experiments (pH 5.0), although

TABLE I  
*Change in Color Intensity Produced by Cysteine Phospho-18-Tungstic Acid  
Reagent with Change in  $\text{HgCl}_2$  Concentration*  
(a) *With Different Amounts of Cysteine Solution Made from One Cysteine  
Hydrochloride Preparation*

	Color standard solution	Test solution
	cc	cc
2 M NaAc	10 (0.4 M)	10
2 " HAc	3 (0.12 M)	3
0.2 M NaOH	2 (0.08 ")	Amount corresponding to that of cysteine solution added
0.01 $\alpha$ M cysteine in 0.2 M HCl	2 ( $4\alpha \times 10^{-4}$ M)	A definite amount for one series; different amounts for other series, as de- scribed
0.01 M $\text{HgCl}_2$	0	Varying amounts
Phospho-18-tungstic acid reagent	4	4
Total volume	50	50

Temperature 24–30°.

TABLE I, *a*—Continued

0.5 cc. cysteine solution				2.0 cc. cysteine solution			
0.01 M HgCl <sub>2</sub> added	$I_x/I_0$		$\alpha$ by Equation 9	0.01 M HgCl <sub>2</sub> added	$I_x/I_0$		$\alpha$ by Equation 9
	Actual determi- nation	By Equation 10			Actual determi- nation	By Equation 10	
cc.				cc.			
0.000	0.238	0.236		0.000	1.000	0.999	
0.100	0.136		0.877	0.20	0.776		0.893
0.160	0.085		0.980	0.40	0.582		0.957
0.200	0.043		0.971	0.50	0.478		0.976
0.225	0.023		0.990	0.60	0.367		0.948
0.25	0.0			0.80	0.155		0.947
0.30	0.0			0.90	0.060		0.957
				1.00	0.0		
				1.50	0.0		
$\alpha$ { Av (Equation 9) 0.956 <sup>±</sup> ± 0.038				. 0.946 ± 0.018			
By Equation 11. 1.046				. . . 0.940			
" " 11-a 0.969				. . . 0.939			
5.0 cc. cysteine solution				10 cc. cysteine solution			
0.01 M HgCl <sub>2</sub> added	$I_x/I_0$		$\alpha$ by Equation 9	0.01 M HgCl <sub>2</sub> added	$I_x/I_0$		$\alpha$ by Equation 9
	Actual determi- nation	By Equation 10			Actual determi- nation	By Equation 10	
cc.				cc.			
0.00	2.493	2.502		0.00	5.032	4.983	
0.51	1.950		0.927	1.05	3.841		0.906
1.00	1.427		0.932	2.03	2.843		0.941
1.50	0.929		0.955	3.00	1.931		0.947
2.01	0.379		0.948	4.00	0.785		0.949
2.20	0.086		0.911	4.53	0.268		0.957
2.40	0.0			4.64	0.141		0.955
2.50	0.0			4.73	0.040		
				5.00	0.0		
$\alpha$ { Av (Equation 9) 0.935 ± 0.014				. 0.942 ± 0.013			
By Equation 11 0.932				. 0.956			
" " 11-a 0.933				. 0.952			

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TABLE I—Concluded

(b) With Same Amounts of Cysteine Solutions Made from Different Cysteine Hydrochloride Preparations

Composition of reaction mixtures, same as in Table I, a

Color standards, 2 cc. of each cysteine hydrochloride solution made from each preparation

Sample 2				Sample 3				Sample 4			
0.01 M HgCl <sub>2</sub> added per 50 cc.	$I_2/I_0$		$\alpha$ by Equation 9	0.01 M HgCl <sub>2</sub> added per 50 cc.	$I_2/I_0$		$\alpha$ by Equation 9	0.01 M HgCl <sub>2</sub> added per 50 cc.	$I_2/I_0$		$\alpha$ by Equation 9
	Actual determination	By Equation 10			Actual determination	By Equation 10			Actual determination	By Equation 10	
cc.				cc.				cc.			
0.00	1.000	1.001		0.00	1.00	0.996		0.00	1.00	1.002	
0.20	0.762		0.840	0.20	0.775		0.889	0.25	0.722		0.899
0.40	0.518		0.830	0.40	0.582		0.957	0.50	0.448		0.906
0.60	0.276		0.829	0.60	0.362		0.940	0.75	0.158		0.891
0.80	0.033		0.827	0.80	0.150		0.941	0.89	0.0		
1.00	0.0			1.00	0.0			1.00	0.0		
2.00	0.0			2.00	0.0			2.00	0.0		
Average. . . 0.8315				. . . 0.932				. . . . . 0.899			
(Equation 9) $\pm 0.0045$				$\pm 0.019$				$\pm 0.005$			
By Equation											
11.... . 0.828				. 0.946				.. . 0.893			
By Equation											
11-a . . . 0.828				. . . 0.941				. . . 0.895			
$\alpha$ By graphical interpolation . . . 0.828				. . . 0.939				. . . 0.890			
Actual determination . . . 0.824				. . . 0.936				. . . . . 0.885			

the precipitation does not take place for several hours at least unless HgCl<sub>2</sub> is present in great excess. If the two substances are added to an acetate buffer containing the phospho-18-tungstic acid reagent, a gradual production of blue color takes place, even before the precipitate is formed. The color production is probably due to the oxidation of intermediate compounds, for instance R-S-OH to R-S-O<sub>2</sub>H and R-S-O<sub>3</sub>H. The increase in

the color intensity of the mixture is practically in linear relation with time in the early stage (20 minutes), and the rate of the intensity increase is roughly in direct proportion to the  $\text{HgCl}_2$  concentration, while the cystine concentration seems to have but little influence upon it.

Cystine is frequently present as an impurity in cysteine preparations, probably as a product of oxidation of the latter during

TABLE II

*Change in Color Intensity Produced by Thioglycolic Acid and Phospho-18-Tungstic Acid Reagent with Change in  $\text{HgCl}_2$  Concentration*

The composition of reaction mixtures was the same as for cysteine, except that 0.01M thioglycolic acid in 0.2M  $\text{HCl}$  was used.

Temperature  $32^\circ \pm 0.2^\circ$ .

2.0 cc thioglycolic acid			
0.01 M $\text{HgCl}_2$	$I_\alpha/I_0$		$\alpha$ by Equation 9
	Actual determination	By Equation 10	
cc			
0.00	1.005	1.001	
0.20	0.796		0.980
0.40	0.596		0.990
0.60	0.387		0.979
0.70	0.293		0.990
0.80	0.195		0.994
0.90	0.093		0.992
0.95	0.040		
0.98	0.010		
1.00	0.0		
$\alpha$	Average (Equation 9)		0.988
	By Equation 11		0.989
	" " 11-a		0.990

preservation. However, in spite of its color production in the mixture containing  $\text{HgCl}_2$  and phospho-18-tungstic acid reagent, it does not interfere with cysteine determination, even when it is present in a high concentration.<sup>5</sup>

By the above method, 94.2 and 94.1 per cent purity, respec-

<sup>5</sup> This is probably due to the slow rate of reaction of  $\text{HgCl}_2$  with cystine compared with that of cysteine.

tively, were obtained for two solutions, one containing  $4\alpha \times 10^{-4}$  M cysteine together with  $20 \times 10^{-4}$  M cystine, and the other containing the same amount of cysteine but no cystine.

*Standardization of Cysteine Hydrochloride Preparations*—As shown in Table I, the amount of cysteine in each preparation is lower than that theoretically expected. That this low content of cysteine is due to the presence of water or cystine will be seen from the results in Table III, which were obtained by the following methods.

*Determination of Water*<sup>a</sup>—As reported by Bergmann and Michalis (10), it was found that cysteine hydrochloride loses not only water and HCl, but also sulfur atoms as  $\text{H}_2\text{S}$ , and becomes

TABLE III  
*Analytical Results of Commercial Cysteine Hydrochloride*

	Sample 1		Sample 2		Sample 3		Theory (R—SH—HCl)	
	per cent	mole in 100 gm	per cent	mole in 100 gm	per cent	mole in 100 gm	per cent	mole in 100 gm
Cysteine	63.77	0.527	72.30	0.597	68.84	0.568	76.85	0.635
Cystine	10.01*	0.042	0	0	0	0	0	0
HCl	21.47	0.587	21.73	0.596	20.83	0.571	23.14	0.635
H <sub>2</sub> O	6.37	0.354	6.01	0.334	10.26	0.570	0	0
Total	101.62*		100.04		99.93		99.99	

\* The somewhat higher result is due to the comparative inaccuracy of the cystine determination

yellowish when dried at  $100^\circ$ . However, it can be freed from water when it is dried in a desiccator over  $\text{P}_2\text{O}_5$  at room temperature under ordinary atmospheric pressure. The loss of water follows an exponential curve when plotted against time, and after 48 hours no loss in weight can be observed. There is no loss of HCl in this drying process, as indicated in the determination of HCl by the following method. For instance, a sample showed HCl content, before and after being dried to constant weight, of 21.47 and 21.51 per cent respectively, and another sample showed 20.83 and 20.79 per cent

<sup>a</sup> Dr Theodore F Lavine of this Institute also obtained satisfactory results (9) by using this method on the author's recommendation

*HCl Determination*<sup>7</sup>—A weighed amount of cysteine hydrochloride is dissolved in a small amount of water, to which a sufficient amount of  $\text{AgNO}_3$  solution is added. The precipitate, which is a mixture of  $\text{AgCl}$  and silver salt of cysteine, is gently boiled after the addition of fuming nitric acid until production of  $\text{CO}_2$  and  $\text{NO}$  has ceased and the liquid part becomes colorless. The solution is diluted with water and  $\text{AgCl}$  is filtered off after the solution is cooled.

*Cystine Determination*—As will be shown in Paper V, cystine can be determined with fairly good results, by the sulfite method.

*Cysteine Determination*—Cysteine contents in percentage were calculated from the values shown in Table I, *a*. All the results obtained by this method are summarized in Table III, their contents being expressed as percentages. The sum of the percentages of all the components is practically 100, which proves that this method of cysteine determination gives accurate results.

#### DISCUSSION AND SUMMARY

It was shown that combination of the reactions shown in Equations 1 and 3 made possible the precise determination of cysteine. The method is, however, limited mostly to the determination of the purity of cysteine preparations, because the presence of substances which rapidly react with  $\text{HgCl}_2$  would upset the quantitative relation. Cystine, which is an occasional impurity in cysteine preparations, was found not to interfere with the determination, which is, no doubt, due to its comparatively slow reaction with  $\text{HgCl}_2$ . Of the various methods of calculating the relative purity ( $\alpha$ ), the graphical method may be the simplest. As it consists of several determinations, the error occurring in the final result is minimized (about  $\pm 0.2$  per cent). As the theoretical consideration of probable errors indicates, the error is large when the amount of  $\text{HgCl}_2$  added is small. If a sufficiently large amount of  $\text{HgCl}_2$  is added, accuracy is obtained by a single determination in which an experimental value is introduced into Equation 9 to obtain the relative purity.

Actual determination of the minimum amount of  $\text{HgCl}_2$  that inhibits the color production of a definite amount of cysteine will

<sup>7</sup> Although  $\text{HCl}$  combined with cysteine can be determined by alkalimetry with methyl red, it seems less accurate than the present method

also give satisfactory results, provided the constant error due to the invisibility of color below  $4 \times 10^{-6}$  M cysteine concentration is introduced (5). However, the method is somewhat tedious when accuracy is desired.

The agreement of the analytical results shown in Table III definitely indicates the applicability of the method for the determination of the purity of cysteine preparations.

The method was shown to be applicable also to thioglycolic acid determination. The experimental procedure is shown under "Technique."

Since commercial cysteine preparations are not pure, before being used as the standard of colorimetric cysteine determination or for other quantitative work they must be accurately standardized by this method. Mere purification is not enough, as water is the principal impurity.

The author would like to express his gratitude to Mr. Robert McNeil of the McNeil Laboratories for his support of this research, and also to Dr. Frederick S. Hammett for his valuable advice.

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# THE DETERMINATION OF THIOL AND DISULFIDE COMPOUNDS, WITH SPECIAL REFERENCE TO CYSTEINE AND CYSTINE

## V. A CRITICAL STUDY OF CYSTINE DETERMINATION BY SULFITE AND PHOSPHO-18-TUNGSTIC ACID REAGENT

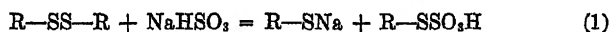
By KAMENOSUKE SHINOHARA\*

(From the Lankenau Hospital Research Institute, Philadelphia)

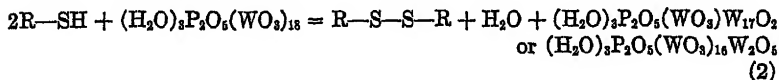
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It was reported in Paper II (1) of this series that cystine can be completely reduced by a strong acid and tin dust, and can be determined with satisfactory accuracy. However, besides being somewhat tedious as a routine method, it was sometimes found, for an unknown reason, to give very low results when applied to urine. As a substitute, therefore, the use of sulfite was studied and found applicable to cystine determination of urine.

The sulfite method was originally used by Folin and Looney (2) and later modified by various workers.<sup>1</sup> The chemistry of the cystine-sulfite reaction, however, was not known until Clarke (4) established Equation 1.



A little later Lugg (3) explained the relation between the color intensity and cysteine or cystine concentration in the presence of sulfite. That the color reaction between cysteine and phospho-18-tungstic acid may be conveniently represented by the following stoichiometrical equation was shown in Paper I by the author (5).



\* Robert McNeil Fellow of the Lankenau Hospital Research Institute.

<sup>1</sup> Lugg's paper (3) is cited here for references to the literature concerning the historical development of this method.



After his critical study, Lugg (6) devised a method for the determination of cysteine and cystine, which is apparently the best of the various sulfite modifications.

However, several important aspects have so far been neglected. They are first, the contribution of color by sulfite alone, which influences the accuracy of the method, especially when the cystine concentration is small; second, a quantitative study of the relation of the color intensity to the sulfite and cystine concentrations; and third, the applicability of  $\text{HgCl}_2$  in the differentiation of various extraneous reducers from cysteine and cystine.

The present paper deals with these aspects, mainly with the purpose of establishing a more reliable method.

#### EXPERIMENTAL

##### *Reagents*

***l*-Cystine solution.** 0.01 M *l*-cystine in 0.2 N HCl (or  $\text{H}_2\text{SO}_4$ ). The cystine is the same preparation as that used in the author's previous work (7). Although hydrolytic decomposition (8) takes place when the solution is left standing at room temperature for a month, and it gives a faint color with the phospho-18-tungstic acid reagent without sulfite (reaction of cysteine), the color intensity produced with sulfite was found to be the same as for a freshly prepared solution. After standing for 2 months, however, the solution was found to produce 99 per cent intensity.

**1 M  $\text{NaHSO}_3$  solution.** This was made from Merck's reagent grade preparation which had been standardized by Volhard's iodine method. The pH of the solution was adjusted roughly to 5.0 by adding 25 cc. of 1 M NaOH solution per liter of the resulting solution. It oxidizes gradually when it is preserved at room temperature (20–32°) with frequent opening of the stopper.<sup>2</sup>

However, it was found that even a 70 day-old solution developed the same intensity as one freshly prepared, either by itself

<sup>2</sup> The decrease in the sulfite concentration within a month, determined by Volhard's iodine method, can be represented within  $\pm 2$  per cent error by Equation 3

$$C = C_0 (1 - 0.0036 N) \quad (3)$$

where  $C$  is the sulfite concentration after  $N$  days and  $C_0$  (1 M) that at the start

or with cystine. The addition of alcohol or brucine was found not to decrease appreciably the rate of this oxidation.

The other reagents, technique, and the color standard employed are the same as described in Papers I and II unless otherwise mentioned. That is, the color development was always carried out in a buffer of 0.4 M sodium acetate and 0.12 M acetic acid (pH 5.0) in a 50 cc. volumetric flask, and the color standard was the solution containing  $4 \times 10^{-4}$  M cysteine.

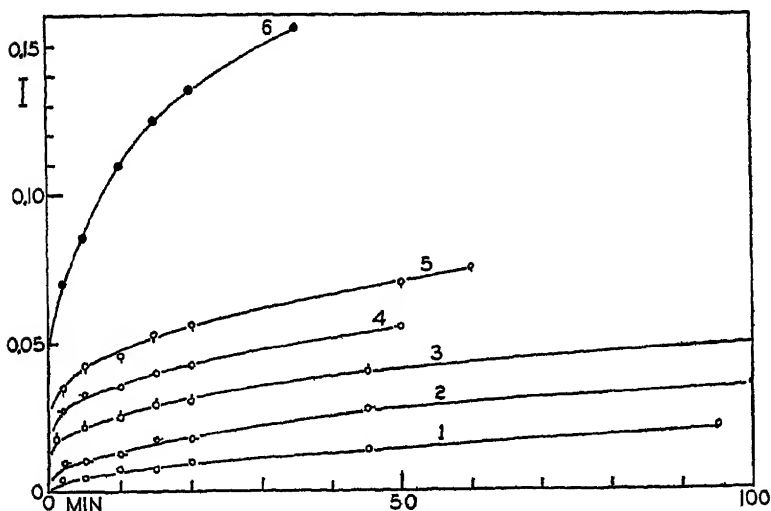


FIG. 1 Color production by  $\text{NaHSO}_3$  and phospho-18-tungstic acid reagent. Composition of color solutions, sodium acetate 0.40 M, acetic acid 0.12 M, sodium sulfite, varying concentrations, phospho-18-tungstic acid reagent 4 cc per 50 cc; total volume 50 cc; temperature 23–24°. Curve 1 denotes 0.02 M  $\text{NaHSO}_3$ , Curve 2 0.04 M, Curve 3 0.06 M, Curve 4 0.08 M, Curve 5 0.10 M, Curve 6 0.20 M.  $I$ , color intensity.

*Color Production by  $\text{NaHSO}_3$  Alone*— $\text{NaHSO}_3$  in contact with the phospho-18-tungstic acid reagent in the specified medium develops blue color so slowly that the maximum is not reached for at least 48 hours. Although experimental conditions, particularly temperature (pH being practically constant), seem to influence the rate of color production, the curves shown in Fig. 1 represent, within the limits of error of colorimeter readings, the

relation of the intensity to the  $\text{NaHSO}_3$  concentration, and time, at 23–28°.

It is seen in Fig. 1 that below 0.1 M  $\text{NaHSO}_3$  there exists, in a practical sense, proportionality between the color intensity and the  $\text{NaHSO}_3$  concentration at any time. The following equation may represent the proportionality at 15 to 20 minutes below 0.1 M  $\text{NaHSO}_3$

$$I = 0.50 C_{\text{NaHSO}_3} \quad (4)$$

where  $I$  is the color intensity and  $C_{\text{NaHSO}_3}$  the concentration of the sulfite.

The presence of  $\text{HgCl}_2$  does not noticeably change the rate of color production.

*Color Production by Cysteine or Cystine in Presence of  $\text{NaHSO}_3$ .*—Two kinds of experiments were carried out, in one of which a definite amount of cysteine or cystine was mixed with varying amounts of  $\text{NaHSO}_3$ , and in the other varying amounts of cysteine or cystine were mixed with a definite amount of  $\text{NaHSO}_3$ . The former experiments may determine the suitable amount of  $\text{NaHSO}_3$  to be used in the latter experiments, which, in turn, give information as to how accurately the color intensity is proportional to the cysteine or cystine concentration. The color intensity here was taken as the difference between that developed in the test solution and that developed in the blank solution by the same amount of  $\text{NaHSO}_3$ .

The results of the first experiments are given in Fig. 2. It shows the following facts. First, cysteine and cystine produce color exponentially<sup>3</sup> with time, and their intensities approach a

<sup>3</sup> The kinetics of the color production seem to be represented by Equation 5

$$k = \frac{2.303}{t \times C_{\text{NaHSO}_3}} \log \frac{I_\infty}{I_\infty - I_t} \quad (5)$$

where  $k$  is the velocity constant in liters per mole per minute, which is roughly 3.00 by calculation;  $t$ , time;  $C_{\text{NaHSO}_3}$ , molar concentration of  $\text{NaHSO}_3$ ;  $I_\infty$  the maximum color intensity developed by cystine (value of the asymptote); and  $I_t$  the intensity developed at time  $t$ . Such an equation may be obtained by assuming that the reaction according to Equation 1 is slow and that according to Equation 2 is very rapid, and that  $C_{\text{NaHSO}_3}$  is so great an excess as used in the experiments remains practically constant

common asymptote within the limits of experimental error, provided their molar concentrations are the same. Second, the extrapolation of the curves to 0 time suggests that cysteine pro-

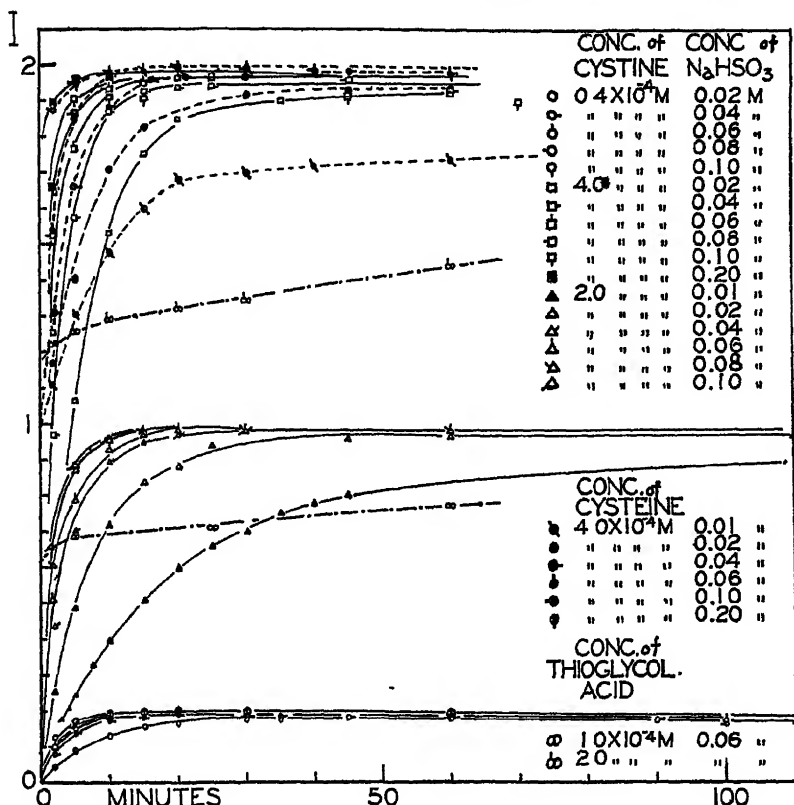


FIG 2 Color production by cysteine and cystine with phospho-18-tungstic acid reagent in the presence of varying amounts of NaHSO<sub>3</sub>. Composition of color solutions, sodium acetate 0.40 M, acetic acid 0.12 M, cysteine or cystine, a definite amount for one series, NaHSO<sub>3</sub>, varying amounts, phospho-18-tungstic acid reagent 4 cc per 50 cc; total volume 50 cc; temperature 21-26° C, color intensity.

duces one-half of the maximum color intensity at the start, while cystine starts at 0 intensity. Third, the maximum intensity reached by cysteine or cystine is twice that reached by cysteine

of the same molar concentration without sulfite, as shown by comparison with Fig. 3 of Paper I (5). Fourth, within a limited time it requires far greater concentration of  $\text{NaHSO}_3$  than that expected from Equation 1 to make the intensity reach practically the maximum (99 per cent).

These facts afford some insight into the theoretical aspect of the color production. That is, the color production by cysteine and cystine in the presence of  $\text{NaHSO}_3$  is due to the reactions expressed by Equations 1 and 2, the former being comparatively slow and the latter very rapid.

Considering the permanency of the maximum color developed, it may be concluded that cysteine-sulfonate is a stable compound under the conditions. The relation between the color intensities and cysteine and cystine concentrations with and without the sulfite can be explained only by Equations 1 and 2. It is of interest to note that cysteine is practically not oxidized beyond the cystine stage.

In devising a practical method for cystine determination, it should be noted that with about 0.06 M  $\text{NaHSO}_3$  concentration the intensity reaches at least 99 per cent of its theoretical value in 15 minutes, beyond which increase in concentration does not materially increase the maximum intensity. The combined intensity developed by the test solutions with sulfite increases with  $\text{NaHSO}_3$  concentration, but the difference between the test and the blank rather decreases.

Based upon the above observations, the second series of experiments was carried out with 0.06 M  $\text{NaHSO}_3$  and varying amounts of cysteine, cystine, or thioglycolic acid, all the other components remaining the same as before. The results show that both cysteine and cystine produce the maximum intensity within 15 minutes; there is no decrease for at least 6 hours, but about 10 per cent after 20 hours at room temperature.

Thioglycolic acid, although it behaves as does cysteine (1) without sulfite, does not produce, within the limited time, twice as much intensity with as without it (Fig. 2). However, the nature of the curve suggests that the difference is merely the rate of reaction for Equation 1, which is probably very slow in the case of thioglycolic acid owing to its molecular structure. If the intensities developed at 15 to 20 minutes by cysteine or by cystine

are plotted against their concentrations, a straight line is obtained, as shown in Fig. 3. The maximum deviation of the observed values from the straight line is about  $\pm 0.02$  unit of intensity ( $4 \times 10^{-6}$  M as cysteine or cystine). The error will, no doubt, be reduced by more careful experimentation.

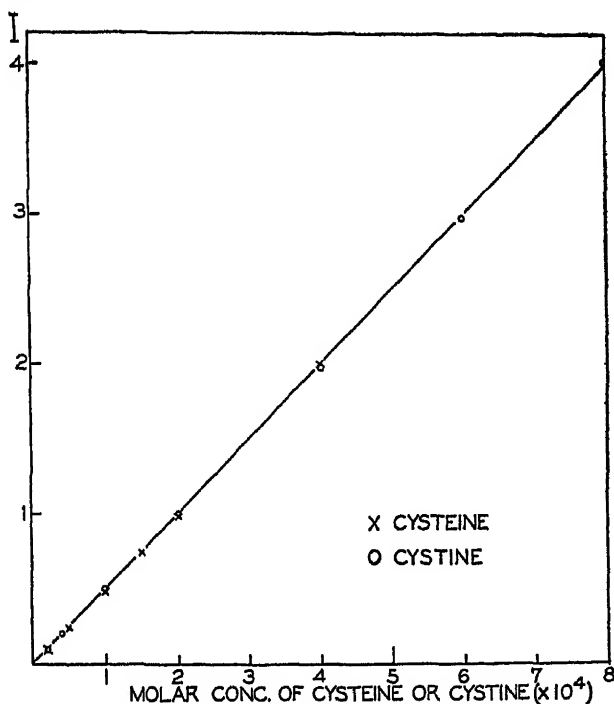


FIG 3 Proportionality of color intensity developed in 15 to 20 minutes by cysteine or cystine with 0.06 M  $\text{NaHSO}_3$  to their concentrations. Composition of color solutions, sodium acetate 0.40 M, acetic acid 0.12 M,  $\text{NaHSO}_3$  0.06 M, cysteine or cystine, varying amounts, phospho-18-tungstic acid reagent 4 to 6 cc. per 50 cc.; total volume 50 cc.; temperature 21–26°.  $I$ , color intensity.

*Color Production by Extraneous Reducers in Presence of  $\text{NaHSO}_3$*   
*Organic*—They produce greater color intensity with  $\text{NaHSO}_3$  than without it. As an exception, resorcinol develops little intensity even with the sulfite, and its color contribution at less than 0.01 M can safely be disregarded. Creatinine, although it produces

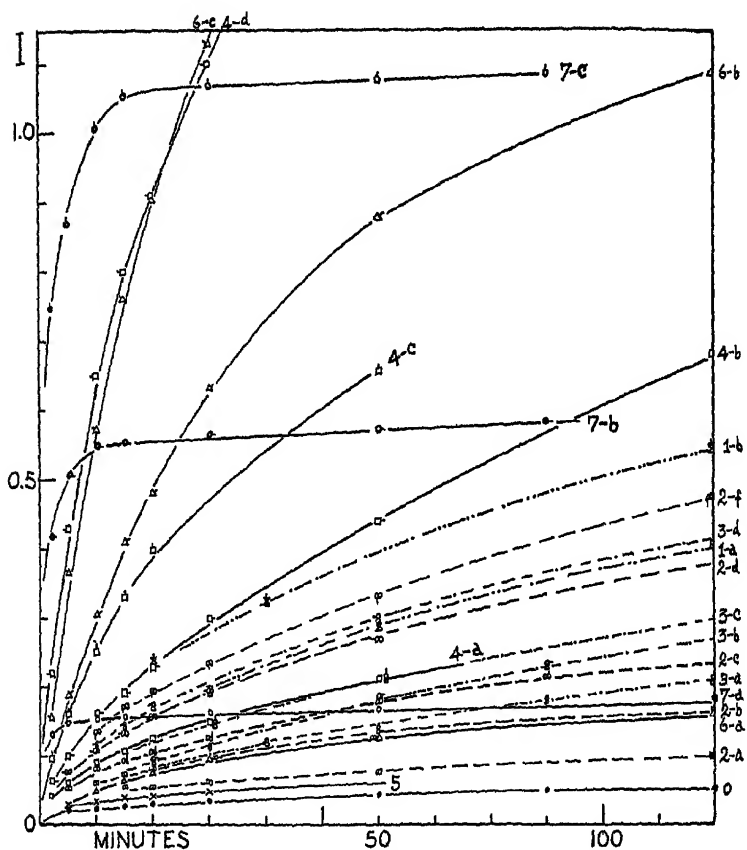


Fig 4. Color production by organic extraneous reducers in the presence of  $\text{NaHSO}_3$ . Composition of color solutions, sodium acetate 0.40 M, acetic acid 0.12 M,  $\text{NaHSO}_3$  0.06 M, extraneous reducer, a varying amount, phospho-18-tungstic acid reagent 4 cc per 50 cc; total volume 50 cc. Color standard,  $4 \times 10^{-4}$  M cysteine in place of an extraneous reducer. Concentrations ( $\times 10^{-4}$  M) of the following solutions are shown by the curves: Curve 0, blank; Curves 1-a, 1-b, 100 and 200 M pyruvic acid; Curves 2-a, 2-b, 2-c, 2-d, 2-f, 10, 20, 40, 100, 200 M creatinine; Curves 3-a, 3-b, 3-c, 3-d, 4, 10, 20, 100 M furfural; Curves 4-a, 4-b, 4-c, 4-d, 1, 2, 4, 20 M catechol; Curve 5, 100 M resorcinol; Curves 6-a, 6-b, 6-c, 0.2, 2.0, 4.0 M quinol; Curves 7-a, 7-b, 7-c, 0.2, 1.0, 2.0 M pyrogallol. I, color intensity.

greater intensity with the sulfite, can also be disregarded below  $4 \times 10^{-4}$  M. Furfural and pyruvic acid give fairly intense color of the same order of magnitude. Catechol develops far more intense color with the sulfite than without it. Quinol with sulfite also develops far greater intensity, without reaching its maximum for a few hours, although it is reached in a few minutes without sulfite. Pyrogallol with sulfite, on the other hand, develops the maximum intensity within 20 minutes, which is the same as that developed by cystine of the same molar concentration.

The details can be seen in Fig. 4 in which the color intensities,  $I$ , represent those directly read. (The intensity of the blank is not subtracted.)

Ascorbic acid, which is a strong reducer, develops the maximum color very rapidly, both with and without sulfite; the intensity is in both cases exactly twice that developed by cysteine of the same molar concentration without sulfite.<sup>4</sup> Ether contributes noticeable color in the presence of sulfite. Even water which has been shaken with an equal volume of ether and filtered through a wet filter also gives color. Repeated washing with water does not reduce the intensity developed by the washings. Chloroform,<sup>5</sup> on the other hand, does not give color at all.

*Inorganic*— $\text{FeSO}_4$  with sulfite develops color very rapidly to its maximum, which is the same as that developed without sulfite (1) if the intensity due to the sulfite alone is subtracted.  $\text{SnCl}_2$  with sulfite gives more color than without, but it does not reach the maximum for at least 2 or 3 hours.  $\text{CuCl}$  with sulfite gives color which increases with time.  $\text{CuCl}_2$  with sulfite neither gives color nor interferes with the cysteine or cystine determination, although it does without sulfite.

*Influence of  $\text{HgCl}_2$  upon Color Development of Cystine in Presence of Sulfite*—It is a well known fact in analytical chemistry (9) that  $\text{HgCl}_2$  forms with  $\text{NaHSO}_3$  a complex sulfonic acid. However, it was found that sufficient  $\text{HgCl}_2$  added to the mixture of cystine and sulfite in the acetate buffer still inhibits the color produc-

<sup>4</sup> Ascorbic acid and glutathione are important constituents of many biological materials; the results with them are reported in Paper VI

<sup>5</sup> This is one of the reasons why ether, as recommended by Lugg (3), should be replaced by chloroform for extracting insoluble thiol compounds, etc., which interfere with cysteine and cystine determination



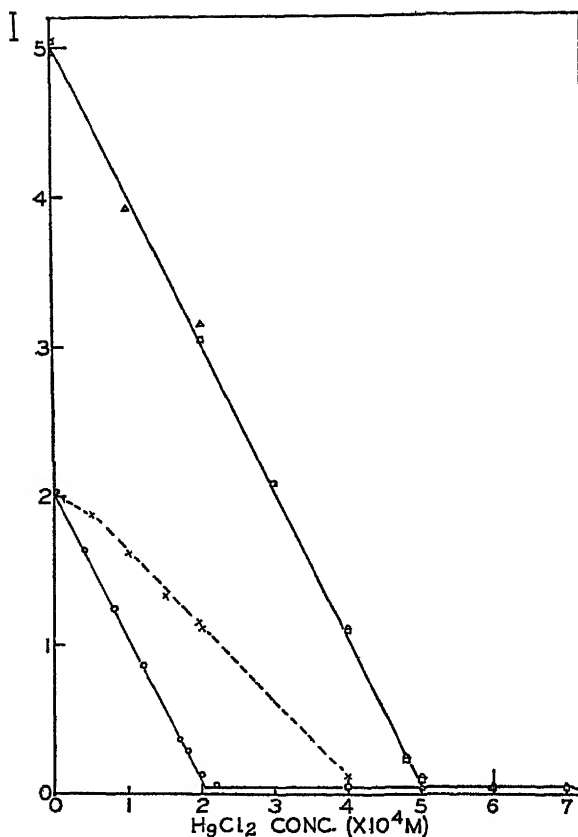


FIG 5 Effect of  $\text{HgCl}_2$  upon the color production of cystine by phospho-18-tungstic acid reagent in the presence of  $\text{NaHSO}_3$ . Composition of color solutions, sodium acetate 0.40 M, acetic acid 0.12 M,  $\text{NaHSO}_3$  0.06 M, cystine  $4 \times 10^{-4}$  M or  $10 \times 10^{-4}$  M,  $\text{HgCl}_2$ , varying amounts, phospho-18-tungstic acid reagent 4 to 6 cc; total volume 50 cc; temperature 24–30°. Color standard,  $4 \times 10^{-4}$  M cystine without sulfite and  $\text{HgCl}_2$  but with all other constituents.  $\Delta$  and  $\square$  represent  $10 \times 10^{-4}$  M cystine,  $\circ$   $4 \times 10^{-4}$  M cystine.  $\times$  represents  $4 \times 10^{-4}$  M cystine; however, in this case the phospho-18-tungstic acid reagent was added immediately after mixing cystine,  $\text{NaHSO}_3$ , and  $\text{HgCl}_2$ . Intensity,  $I$ , is that directly observed (the blank is not subtracted).

tion, provided there is an interval of more than 15 minutes before the addition of the phospho-18-tungstic acid reagent. If, on the other hand, the reagent is added immediately after the mixing,

intense color is produced. The reason is that a part of the cysteine produced from the cystine and sulfite reacts with  $\text{HgCl}_2$ , and the other part with the reagent. Thus the effect of  $\text{HgCl}_2$  upon the color production of cystine in the presence of sulfite was studied by adding the reagent 20 minutes after the three substances were mixed in the buffer solution, the rest of the procedure being carried out as previously described.

The experimental results shown in Fig. 5 distinctly indicate that the color intensity decreases linearly with increase in  $\text{HgCl}_2$  concentration until the amount of  $\text{HgCl}_2$  exceeds the equivalent amount of cystine, beyond which the intensity remains constant and corresponds to that given by the same amount of sulfite alone.

The relation between intensity and  $\text{HgCl}_2$  concentration may be expressed by Equation 6<sup>6</sup>

$$\frac{I_x}{I_0} = \frac{2}{\alpha} \left( \beta \frac{C'_a}{C'_0} - \frac{2}{C'_0} C_{\text{Hg}} \right) \quad (6)$$

where  $I_x$  is the intensity developed in a solution to which  $C'_a$  moles per liter of cystine (or cysteine), calculated on the assumption that the cystine (or cysteine) preparation used is 100 per cent pure, and  $C_{\text{Hg}}$  moles per liter of  $\text{HgCl}_2$  are added.  $\beta$  is the relative purity of the cystine (or cysteine) preparation.  $I_0$  and  $C'_0$  are respectively the color intensity and molar concentration of the cysteine of the color standard, calculated on the assumption that the cysteine preparation used as the standard is 100 per cent pure.  $\alpha$  is the relative purity of the cysteine used as the standard.

This equation differs from the one applied to cysteine, shown in Paper IV, mainly by the additional factor 2. This evolves from the fact that 1 mole of cystine (or cysteine) develops twice the color intensity with sulfite as without it.

Although, theoretically, this method can also be used for standardizing cystine preparations, the complexity of the reactions involved and the color given by sulfite alone make it somewhat less precise than in the case of cysteine, and it seems to have no advantage over direct color comparison between cystine with sulfite and cysteine of known purity without sulfite.

<sup>6</sup> To clarify the establishment of such an equation, see Paper IV of this series (10)

The extraneous reducers described above produce practically the same color intensity in the presence of  $\text{HgCl}_2$  as in its absence, no reduction of  $\text{HgCl}_2$  by catechol, pyrogallol, etc., having been observed. Ascorbic acid can be accurately differentiated from cysteine and cystine. On the other hand, formaldehyde in the presence of sulfite not only interferes with the color production by these extraneous reducers, but also produces an immediate precipitation with urine, thus preventing colorimetry. For these reasons,  $\text{HgCl}_2$  should be substituted for  $\text{HCHO}$  for the differentiation of extraneous reducers from cysteine and cystine in the presence of sulfite.

*Method for Determination of Cystine*—As a result of the critical studies mentioned above, a new method for cystine determination is established. The procedure is as follows:

To each of two 50 cc. volumetric flasks add 10 cc. of 2 M sodium acetate, 3 cc. of 2 M acetic acid, 3 cc. of 1 M sodium bisulfite solution, prepared as described under "Reagents," and an amount of NaOH, or preferably LiOH, just sufficient to neutralize the acid contained in the solution to be tested, followed by a definite amount of the test solution. At this point, to one of the mixtures add sufficient (4 cc.) phospho-18-tungstic acid reagent and make up the total volume to exactly 50 cc. by adding water (test color solution). The color intensity developed is measured in 15 to 20 minutes against the cysteine standard described in Paper II ( $4 \times 10^{-4}$  M cysteine in the acetate buffer without sulfite).

To the other flask add 3 cc. of 0.1 M  $\text{HgCl}_2$  solution, and set the resulting mixture aside. After 20 minutes proceed just as in the case of the test color solution (blank color solution).

If the intensity of the test color solution is  $I_t$  and that of the blank color solution  $I_b$ , then the difference,  $I_t - I_b$ , is the intensity given by cysteine and cystine.

Therefore, the combined molar concentration of cysteine and cystine in the colored test solution can be obtained by Equation 7

$$C_{\text{R-SH}} + C_{\text{R-S-S-R}} = C_0 \frac{I_t - I_b}{2} \quad (7)$$

where  $C_{\text{R-SH}}$  and  $C_{\text{R-S-S-R}}$  are the respective concentrations of cysteine and cystine in the colored test solution, and  $C_0$  the molar concentration of cysteine in the standard (in the author's experiment,  $4 \times 10^{-4}$  M).

TABLE I  
*Determination of Cystine Mixed with Cysteine*

Cysteine added	Cystine added	Cysteine found	Cystine found
$\times 10^4 M$	$\times 10^4 M$	$\times 10^4 M$	$\times 10^4 M$
4 0	0	4 01	0.0
4 0	0 1	4 00	0 110
4 0	0 2	4 02	0 210
4 0	0 4	4 01	0.385
4 0	1 0	3 99	0.978
4 0	2 0	3 99	2 060

TABLE II  
*Determination of Cystine in Presence of Extraneous Reducers by Sulfite Method*

	Extraneous reducers added	Cystine added	Cystine found	Difference
	$\times 10^4 M$	$\times 10^4 M$	$\times 10^4 M$	$\times 10^4 M$
Pyruvic acid	227	4 0	3.75	-0 25
	227	1 0	0 85	-0 15
	227	0 4	0 33	-0 07
	227	0 2	0 18	-0 02
Creatinine	100	4 0	3.72	-0 23
	100	1 0	0 83	-0 17
	100	0 4	0 30	-0 10
Furfural	100	4 0	3 75	-0 25
	100	1 0	0 90	-0 10
	100	0 4	0 38	-0 02
Pyrogallol	10	4 0	3 45	-0 55
	10	0 4	1 17	+0 77
	6	4 0	3 54	-0 46
	6	1 0	0 97	-0 03
Catechol	6	0 4	0 405	+0 005
	100	4 0	3 06	-0 94
	100	1 0	0 995	-0 004
	100	0 4	0 375	-0 025
Resorcinol	100	4 0	3 95	-0 05
	100	1 0	1 01	+0 01
	100	0 4	0 405	+0 005
	100	0 1	0 095	-0 005

The cystine concentration is obtained by subtracting from the above equation the cysteine concentration which has been found by the method described in Paper II (1).

The preparation of the original test solution requires a different procedure, depending upon how and from what it is prepared. Shaking it with chloroform, as mentioned in Paper II, will help to eliminate some interfering substances.

By combining the method of cysteine determination described in Paper II (1) with the present method, cystine mixed with cysteine can be satisfactorily determined. Table I shows one example of results obtained by carrying out the two methods in a routine manner.

The accuracy of the present method when applied to solutions containing extraneous reducers can be seen from Table II, the results in which were obtained by carrying out the method in a routine manner.

The concentrations of the extraneous reducers are very high. Such extreme conditions can hardly exist in actual cases, and yet the method gives fairly satisfactory accuracy, which will undoubtedly be greatly increased by practise.

The applicability of this method to urine will be shown in Paper VII.

#### SUMMARY

The conditions involved in the color production by cysteine or cystine and phospho-18-tungstic acid reagent in the presence of  $\text{NaHSO}_3$  have been investigated, and a method for the determination of cystine has been devised

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## THE DETERMINATION OF THIOL AND DISULFIDE COMPOUNDS, WITH SPECIAL REFERENCE TO CYSTEINE AND CYSTINE

### VI. THE REACTIONS OF ASCORBIC ACID AND GLUTATHIONE WITH PHOSPHO-18-TUNGSTIC ACID REAGENT

BY KAMENOSUKE SHINOHARA\* AND KIVELY E. PADIS

*(From the Lankenau Hospital Research Institute, Philadelphia)*

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Ascorbic acid, a strong reducer and a normal constituent of urine and other biological materials, is probably the most important substance to be considered when a method for cysteine and cystine determination based upon the reductivity of cysteine is applied to these materials. Glutathione, glutamylcysteinylglycine, also a frequent constituent of many biological materials, is another compound whose behavior toward phospho-18-tungstic acid must be carefully examined. Experimentation along these lines showed that ascorbic acid can be differentiated from cysteine and cystine, and any one can be quantitatively determined in a mixture of them. Glutathione, however, showed peculiar behavior in the test, which can probably be attributed to its hydrolysis. Thus, when cysteine or cystine coexists with glutathione, the method is useless, unless glutathione is determined as cysteine after hydrolysis with an acid.

#### EXPERIMENTAL

##### *Reagents*

0.01 M ascorbic acid in 0.2 N  $\text{H}_2\text{SO}_4$ . Merck's preparation (cecion or crystalline vitamin C) was used without further analysis for its purity, except that the water content was determined by drying over  $\text{P}_2\text{O}_5$  and found to be negative. It is highly probable that the preparation is at least 98 per cent pure according to the

\* Robert McNeil Fellow of the Lankenau Hospital Research Institute

color intensity developed with a freshly prepared solution and the phospho-18-tungstic acid reagent, as will be shown later. The rapid oxidation of the solution on standing at room temperature can also be detected by the decrease in the color intensity developed by the solution with the reagent. For this reason, the solution was used no more than 1 hour after its preparation or its strength was redetermined if used.

0.01 M glutathione in 0.2 M HCl. Eastman Kodak Company's and Hoffmann-La Roche's preparations were used without further analysis. The water content of the former is unknown and that of the latter was found to be 0.7 per cent by drying it over  $P_2O_5$ . The solution changes fairly rapidly on standing at room temperature, as will be shown later.

Other reagents and experimental technique are exactly the same as previously stated (1-3) unless otherwise mentioned. The color standard contains  $4 \times 10^{-4}$  M cysteine with the specified amount of the other constituents (2).

#### *Ascorbic Acid*

*Change of 0.01 M Ascorbic Acid in 0.2 N  $H_2SO_4$* —The solution, when tested for its color production with phospho-18-tungstic acid in the specified medium immediately after its preparation, gives twice the intensity produced by cysteine of the same molar concentration. However, the ability to produce color decreases rapidly as the solution stands in the air at room temperature.

Table I shows roughly the rate of this change. The solution when preserved in a nitrogen atmosphere seems to remain unchanged.

*Color Production of Ascorbic Acid with Phospho-18-Tungstic Acid Reagent*—Like cysteine, ascorbic acid very rapidly reduces the complex acid reagent in the specified acetate-acetic acid buffer to produce blue color. The following experimental results are shown in Fig 1: first, the time interval required for the attainment of the maximum intensity increases with increase in the ascorbic acid concentration; second, the color intensity is directly proportional to the ascorbic acid concentration; third, the maximum color intensity developed by ascorbic acid is twice that developed by cysteine of the same molar concentration, which conforms with the fact that 2 hydrogen atoms are lost in its oxidation (4).

Addition of HCHO somewhat decreases the rate of color production. One peculiar phenomenon for which no explanation is found is that when 1 cc. of 38 per cent (12.67 M) HCHO solution is added to the mixture containing a definite amount of ascorbic acid, just as in the case of other extraneous reducers (2) the rate of color production and also the maximum intensity seem to diminish, while when 6 cc. of 2 M HCHO solution (the same amount as in the solution above) are added, the rate and the maximum intensity are the same as without HCHO (Fig. 1). Principally for this reason, the quantitative differentiation of cysteine and ascorbic acid should be carried out with 6 cc. of

TABLE I

*Change of 0.01 M Ascorbic Acid in 0.2 N H<sub>2</sub>SO<sub>4</sub> on Standing in Air at 25–29°, Indicated by Change in Color Intensity Developed by Phospho-18-Tungstic Acid*

Composition of the color solutions, sodium acetate 0.40 M, acetic acid 0.12 M, ascorbic acid  $4 \times \alpha \times 10^{-4}$  M (=2 cc. of 0.01  $\alpha$  M solution in 0.2 N H<sub>2</sub>SO<sub>4</sub> per 50 cc.), phospho-18-tungstic acid reagent 4 cc. per 50 cc.; total volume 50 cc.

The color standard was a solution containing  $4 \times 10^{-4}$  M cysteine in place of ascorbic acid

$\alpha$  = relative purity of ascorbic acid which changes on standing

Color intensity	Time, hrs								
	0.05	0.17	21	22	25	28	45.5	124	336
Solution A . . .		1.96		1.54		1.45	0.992	0.072	0.0
" B. . .	1.97		1.53		1.39				

2 M HCHO solution instead of 1 cc. of 38 per cent solution, and the intensity determined after about 15 minutes. By employing this method of differentiating cysteine and ascorbic acid, they were determined in mixtures of various ratios. The results in Table II show the applicability and accuracy of the method.

*Color Production by Ascorbic Acid in Presence of NaHSO<sub>3</sub>*—Ascorbic acid produces the same color intensity with NaHSO<sub>3</sub> as without it, provided the intensity contributed by NaHSO<sub>3</sub> is subtracted from that observed. Also, the intensity is strictly proportional to the concentration, as seen in Fig. 2. Ascorbic acid, after being oxidized by the introduction of air, produces no color at all, even in the presence of NaHSO<sub>3</sub>.



## 700 Thiol and Disulfide Compounds. VI

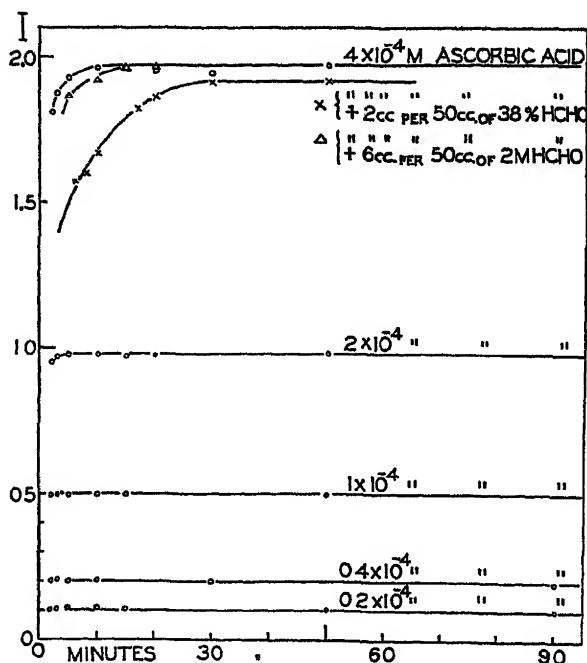


FIG. 1. Color production of ascorbic acid with phospho-18-tungstic acid reagent. Composition of color solutions, sodium acetate 0.4 M, acetic acid 0.12 M, ascorbic acid in varying amounts, phospho-18-tungstic acid reagent 4 cc per 50 cc; total volume 50 cc; temperature 26–32°. For a color standard a solution of the same composition was used, except that it contained  $4 \times 10^{-4}$  M cysteine in place of ascorbic acid. I, color intensity.

TABLE II  
Determination of Cysteine and Ascorbic Acid in Mixture by Use of HCHO

Cysteine added	Ascorbic acid added	Cysteine found	Ascorbic acid found
$M \times 10^4$	$M \times 10^4$	$M \times 10^4$	$M \times 10^4$
2.0	0.0	2.04	0.0
0.0	3.22	0.0	3.22
2.0	0.805	2.0	0.81
2.0	1.33	2.02	1.19
2.0	0.264	1.98	0.25
0.4	1.33	0.44	1.12
0.4	0.665	0.44	0.56
0.4	1.80	0.424	1.82

TABLE III  
Determination of Cysteine or Cystine and Ascorbic Acid in Mixture by Use of  $HgCl_2$

	Cysteine or cystine added	Ascorbic acid added	Cysteine or cystine found	Ascorbic acid found
	$M \times 10^4$	$M \times 10^4$	$M \times 10^4$	$M \times 10^4$
Cysteine added	0.0	2.08	0.0	2.08
	0.2	2.08	0.142	2.07
	0.2	2.38	0.04	2.41
	0.4	1.04	0.39	0.996
	2.0	0.208	1.94	0.212
	2.0	0.52	1.92	0.514
	4.0	0.12	3.92	0.086
Cystine added	0.215	1.00	0.160	1.03
	0.225	2.38	0.06	2.41
	0.860	1.00	0.77	1.01
	2.15	0.20	2.11	0.205
	4.3	0.50	4.30	0.46

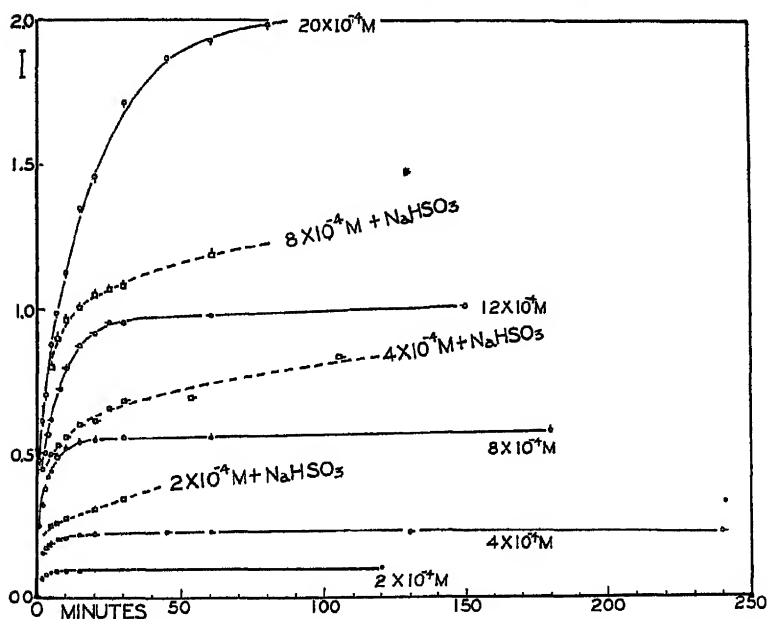


FIG 2 Color production of glutathione with phospho-18-tungstic acid reagent with and without  $NaHSO_3$ . Composition of color solutions, sodium acetate 0.4 M, acetic acid 0.12 M, glutathione in varying amounts, as shown on the figure, phospho-18-tungstic acid reagent 4 cc per 50 cc; total volume 50 cc. The presence or absence of  $NaHSO_3$  is indicated on the curves. I, color intensity.

$\text{HgCl}_2$  produces a dark precipitate with ascorbic acid in the acetate buffer, both with and without sulfite, which is very likely due to the former's reduction by the latter. However, if the phospho-18-tungstic acid reagent is added 1 minute after mixing the other components, practically the same color intensity is developed and the precipitate, although present, scarcely interferes with the colorimetry unless the ascorbic acid concentration is higher than  $1.5 \times 10^{-5}$  M. The rate of color production is not noticeably changed in the presence of 0.06 M  $\text{HgCl}_2$ , the concentration used for differentiating cysteine and cystine from extraneous reducers.

Cysteine or cystine and ascorbic acid, when mixed, can be determined with fair accuracy by the method for cystine determination unless the amount of the former is very small in comparison with the latter, as shown in Table III. In spite of the presence of the keto group in its molecule, ascorbic acid was found not to give red color with nitroprusside by the modified nitroprusside test (5).

#### *Glutathione*

As briefly pointed out in Paper I, a sample of glutathione (Eastman Kodak Company) showed various peculiarities in its color production with phospho-18-tungstic acid reagent, which were at that time considered for several reasons to be due to the oxidized glutathione produced during preservation. However, later Hoffmann-La Roche's preparation, for which high purity was claimed, was obtained and showed the same peculiarities. Both samples showed by the modified nitroprusside test (5) intense red color which is probably greater than that produced by cysteine of the same molar concentration.

The agreement of two samples of different manufacturers in connection with the intense nitroprusside reaction indicates that the peculiarities are due to reduced glutathione, not oxidized, though a strict analytical test proving this point is lacking, owing simply to the scarcity of the material. Since these points undoubtedly throw light on the chemistry of glutathione and on the establishment of an analytical method for its determination, they will be reported.

#### *Color Production of Glutathione with Phospho-18-Tungstic Acid*

*Reagent*—The color production by glutathione in the acetate buffer is greatly influenced by the order of addition; that is, when the reagent is added before glutathione, the apparent maxi-

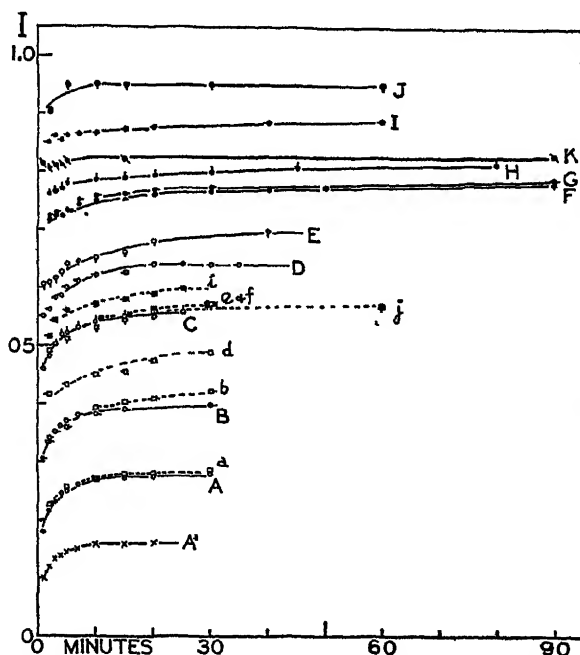


FIG. 3 Change in color production with phospho-18-tungstic acid reagent of 0.01 M glutathione solution in 0.2 M HCl on standing. Temperature 25–33.5°. Color intensities,  $I$ , were developed in the same way as the cysteine color standard solution containing  $4 \times 10^{-4}$  M cysteine. Glutathione from the Eastman Kodak Company is denoted by the capital letters. A' 0.13 hour old (the reagent was added before glutathione), A 0.13 to 2.0 hours old, B 23 hours, C 48 hours, D 70 hours, E 93 hours, F 117 hours, G 144 hours, H 168 hours, I 240 hours, J 288 hours, K 360 hours. Glutathione from Hoffmann-La Roche, Inc., is indicated by the lower case letters. a 0.1 to 1.0 hour old, b 18 to 21 hours, d 66 hours, e 90 hours, f 114 hours, i 240 hours, j 282 hours

imum intensity reached in about 10 minutes is roughly one-half of that developed when the order is reversed (Fig. 3). Hence, in the further experiments the latter order was employed, in conformity with the case of cysteine. The rate of color develop-

ment decreases with increase in glutathione concentration, and the intensity is not directly proportional to the concentration. Moreover, the intensity does not reach, in a strict sense, its maximum, but gradually increases with time. The apparent maximum intensity developed, say in 1 hour, is far less than by cysteine of the same molar concentration. These aspects can be seen in Fig. 2.

The addition of the specified amount of HCHO totally inhibits the color production, as is the case with cysteine.

With  $\text{NaHSO}_3$  glutathione develops greater intensity than without, the intensity reaching its maximum more slowly. Therefore the relation between intensity and concentration cannot be known.  $\text{HgCl}_2$  added in the same manner as for cystine entirely prevents color production by glutathione in the presence of  $\text{NaHSO}_3$ . From this it can safely be inferred that oxidized glutathione does not give color with  $\text{NaHSO}_3$  when  $\text{HgCl}_2$  is added.

*Change of Glutathione Solution on Standing*—Some chemical change takes place in a glutathione solution preserved at room temperature. It can be detected when a certain amount of the solution is tested at various intervals for its color production with phospho-18-tungstic acid reagent under the conditions of the cysteine determination. The change in the mode of color production by glutathione solutions of different ages is seen in Fig. 3. A striking aspect is that the color intensity increases with the age of the solution. 0.01 M glutathione solution in 0.2 M HCl changes far more rapidly than that to which no acid has been added. The difference between the two preparations, as seen in Fig. 3, is probably due to the conditions under which the two experiments were carried out, the Eastman Kodak Company's preparation being tested exactly 1 year before the Hoffmann-La Roche. However, the results with the two preparations coincide well within 24 hours (Fig. 3). It is difficult to ascertain the final intensity produced by the solutions on standing, owing probably to oxidation of the original preparation and its product, yet the intensity produced with a 288 hour-old solution is 95 per cent of that expected from the molar concentration of the  $-\text{SH}$  group.

*Change of Glutathione at High Temperature ( $80^\circ \pm 0.1^\circ$ )*—In order to study more accurately the change that takes place on aging of the glutathione solution, four series of experiments were

carried out in which the change was accelerated by heating to  $80^{\circ} \pm 0.1^{\circ}$  in a nitrogen atmosphere. The apparatus used is the same as in the hydrolysis of cystine (5). Four 0.01 M glutathione solutions were used, the first containing 1.0 M HCl, the second 0.2 M HCl, the third neither acid nor alkali, and the fourth 0.2 M NaOH.

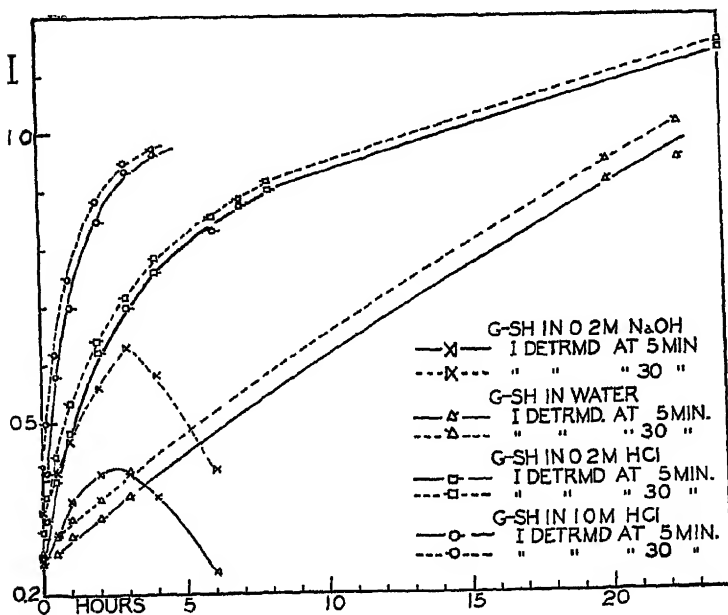


FIG. 4. Change of glutathione solutions at  $80^{\circ}$  indicated by color production with phospho-18-tungstic acid reagent. Composition of color solutions, sodium acetate 0.40 M, acetic acid 0.12 M, 0.01 M glutathione solution 2 cc per 50 cc, phospho-18-tungstic acid reagent 4 cc per 50 cc.; total volume 50 cc.  $I$ , color intensity

All of the solutions were found to undergo the change at a much higher rate than at room temperature, but the rates differ. As shown in Fig. 4, the change is more rapid, the more strongly acid the solution is. Some of the curves show more than the unit intensity expected for the amount of  $-SH$  group used. This is, no doubt, due to the evaporation of water during heating.

In fact, the volume of the solution left after 24 hours was found to be only 50 cc., while it should be 75 cc.

In all these solutions, production of  $\text{H}_2\text{S}$  is observed when tested by lead acetate paper inserted in a tube through which  $\text{N}_2$  gas from the solution is passed. Within 30 minutes, however, it seems that a solution higher in acidity is slower in  $\text{H}_2\text{S}$  production.

Glutathione in 0.2 M NaOH changes as rapidly as that in 0.2 M HCl at first, but another decomposition also takes place rapidly. The solution becomes brownish in 10 minutes, and shows marked reaction of  $\text{H}_2\text{S}$ . The manner in which the heated alkaline solution produces color in the acetate buffer greatly differs from that of the acid solutions in that the color production by the alkaline solution does not reach the apparent maximum for several hours. This behavior is probably due to the presence of a large amount of  $\text{H}_2\text{S}$ . Owing to this rapid side reaction, its curve (Fig. 4) is quite different from those of the acid solutions.

#### DISCUSSION

With this experimental evidence, the method of determining cysteine can also be applied to the determination of ascorbic acid, provided other extraneous reducers like quinol, pyrogallol, ferrous salts, cuprous salts, etc., are absent, cysteine being used as the standard. The advantage of this method is that it enables simultaneous determination of cysteine, cystine, and ascorbic acid from each other by the use of  $\text{HCHO}$  and  $\text{HgCl}_2$ . Such a method probably has wide application for biological materials. Even qualitatively the modified nitroprusside test for cysteine (5) fails to produce color in the presence of ascorbic acid.  $\text{HgCl}_2$  was reported to oxidize ascorbic acid (6) and slight reduction of  $\text{HgCl}_2$  was observed also in the present experiments, but under the conditions of the cysteine determination this seems not to interfere seriously with accuracy.

Glutathione reduces the phospho-18-tungstic acid reagent only partially and even the apparent maximum intensity slowly increases. Experiments on the change of the glutathione indicate that the change is catalyzed by acid and also by base, like the hydrolysis of sucrose. However, in alkaline solution a side reaction in which  $\text{H}_2\text{S}$  is produced takes place to such an extent that after a certain point the change is obscured. This change of

glutathione is very likely due to its hydrolysis, producing either cysteine or its dipeptide. On account of these peculiarities cysteine or cystine cannot be determined when glutathione is mixed with them. However, as experimental results show, glutathione heated at 80° for 5 hours in 1 N HCl (or probably H<sub>2</sub>SO<sub>4</sub>) will produce practically the same intensity<sup>1</sup> as cysteine of the same molar concentration, and hence cysteine and glutathione can be determined as cysteine after hydrolysis, when they are mixed in a test material.

Hunter and Eagles (8) reported that high alkalinity caused by the addition of a slight excess of NaOH to test solutions of glutathione gave the same intensity as cystine of the same molar concentration in the presence of Na<sub>2</sub>SO<sub>3</sub>. Whether this is due to the shift of reduction potential of glutathione or its decomposition cannot be answered at present.

The establishment of a method for determining glutathione awaits further research. At present the modified nitroprusside test (5) will give fairly dependable results, although the precision is very low. Ascorbic acid and glutathione can easily be differentiated by the use of HCHO, as stated before.

With regard to the incomplete reduction of the reagent by glutathione, Green (9) reported the reduction potentials of cysteine, glutathione, and ascorbic acid. However, a definite answer awaits the study of the oxidation-reduction potential of the phospho-18-tungstic acid reagent.

#### SUMMARY

The behavior of ascorbic acid and glutathione toward the phospho-18-tungstic acid reagent was studied. Ascorbic acid can be both differentiated from cysteine and cystine and also determined by the same method, provided other extraneous reducers are absent. Glutathione produces far less intensity than cysteine of the same molar concentration. A solution hydrolyzes on standing to give more intense color with the phospho-18-tungstic acid reagent. The hydrolysis is catalyzed by acids and bases. This peculiarity of glutathione limits the determination

<sup>1</sup> The production of H<sub>2</sub>S in acid solution after 5 hours is negligible. Concerning the error in cysteine and cystine determinations by hydrolysis, Lugg's paper is cited (7).



of cysteine and cystine unless the glutathione is previously hydrolyzed and determined as cysteine.

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## THE DETERMINATION OF THIOL AND DISULFIDE COMPOUNDS, WITH SPECIAL REFERENCE TO CYSTEINE AND CYSTINE

### VII. APPLICATION OF THE MODIFIED PHOSPHO-18-TUNGSTIC ACID METHOD FOR THE DETERMINATION OF CYSTEINE, CYSTINE, AND ASCORBIC ACID IN URINE

By KAMENOSUKE SHINOHARA\* AND KIVELY E. PADIS

*(From the Lankenau Hospital Research Institute, Philadelphia)*

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The phospho-18-tungstic acid methods modified by one of the authors (1-6) for the determination of cysteine and cystine were shown to give satisfactory results when applied to pure solutions, even in the presence of various extraneous reducers. The advantage of these methods is that by them not only can cysteine and cystine be differentiated but also ascorbic acid from either of them. One limitation is that the presence of glutathione upsets the determination unless it is hydrolyzed and determined as cysteine.

To study the applicability of the methods to urine a series of experiments was carried out, during which the details of the methods had to be modified slightly, owing to the chemical complexity of urine. The results show that the method can be applied to urine with satisfactory accuracy for the purposes mentioned above. The determination of the substances in fresh normal urine showed that it usually contains cystine and ascorbic acid.

#### EXPERIMENTAL

*Color Production by Cysteine Added to Urine*—Fresh urine gives fairly intense color in the modified phospho-18-tungstic acid test (1). Therefore, that used in the present experiment was previously oxidized by bubbling air vigorously through it. The oxidation is complete in 3 hours at most, as is seen in Fig. 1

\* Robert McNeil Fellow of the Lankenau Hospital Research Institute

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which shows the decrease in intensity produced by 10 cc. of urine in the phospho-18-tungstic acid test for cysteine.

10 cc. of the oxidized urine, to which varying amounts of 0.01 M cysteine in 0.2 M HCl had been added, were put in a buffer of 0.4 M sodium acetate and 0.12 M acetic acid and the rest of the process followed exactly the same as with pure cysteine solution (1).

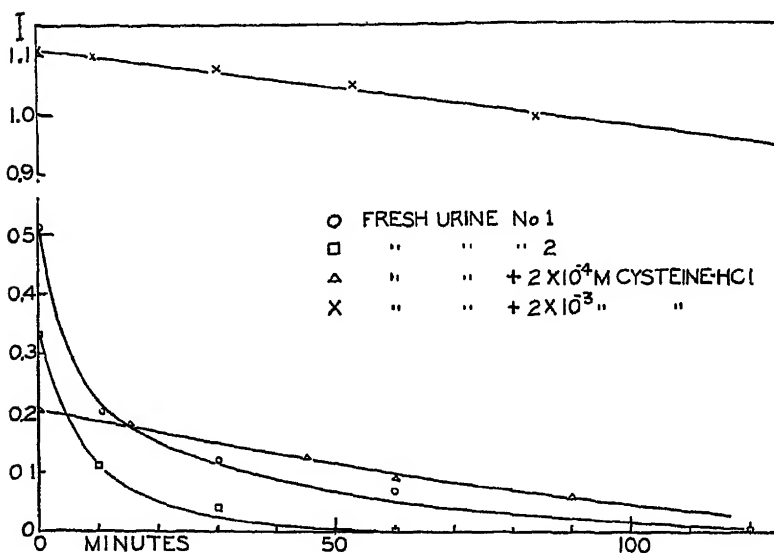


FIG. 1. Oxidation of urine by air, indicated by color intensity produced in phospho-18-tungstic acid test for cysteine. Temperature 25–28° (air is vigorously bubbled through the urine). Composition of test solutions, sodium acetate 0.4 M, acetic acid 0.12 M, urine 10 cc. per 50 cc., phospho-18-tungstic acid reagent 4 cc. per 50 cc.; total volume 50 cc. Color standard,  $4 \times 10^{-4}$  M cysteine and an adequate amount of brom-thymol blue solution in place of urine. I, color intensity.

The color standard used here contained an adequate amount of brom-thymol blue solution, added to simulate the yellow color of the urine, besides  $4 \times 10^{-4}$  M cysteine and other components. The experimental results are given in Fig. 2 which shows that the color development with time is practically the same as when pure cysteine is used, except that here the intensity slightly increases

with time, even after 30 minutes. This may be due to the presence of some extraneous reducers (like creatinine) even after oxidation, which is indicated by the fact that the blank which has HCHO in it produces faint color on standing. The intensity developed after 15 minutes by urine containing various amounts of cysteine is proportional to the cysteine concentration, provided

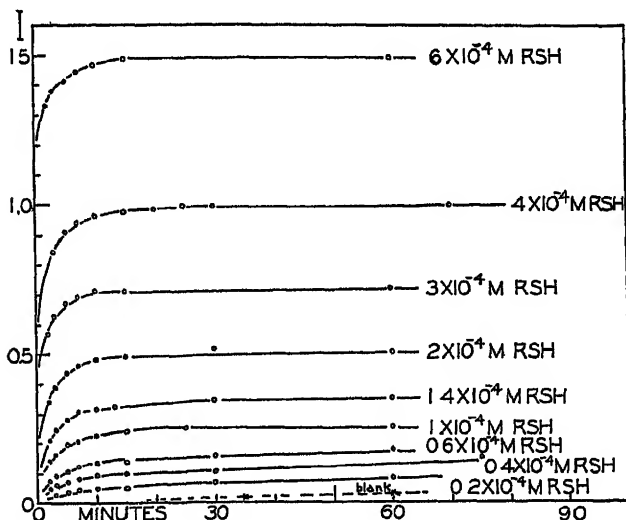


FIG 2 Color production of cysteine in urine with phospho-18-tungstic acid reagent. Composition of colored test solutions, sodium acetate 0.4 M, acetic acid 0.12 M, urine containing varying amounts of cysteine 10 cc. per 50 cc., phospho-18-tungstic acid reagent 4 cc. per 50 cc.; total volume 50 cc. Color standard, solution containing  $4 \times 10^{-4}$  M cysteine and an adequate amount of brom-thymol blue besides all other components of the test solution. Blank, solution containing the urine to which no cysteine is added. The presence of HCHO does not change the intensity. I, color intensity.

the intensity given by the blank is subtracted from that observed, especially when the cysteine concentration is small. It was found that addition of 6 cc. of 2 M HCHO totally inhibits the color production of cysteine regardless of its concentration (see Table I).

These experimental results indicate that cysteine can be determined in urine with rather high accuracy by the method.

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*Color Production by Cystine Added to Urine*—The oxidized urine containing varying amounts of  $0.01\text{ M}$  cystine in  $0.2\text{ M}$   $\text{HCl}$

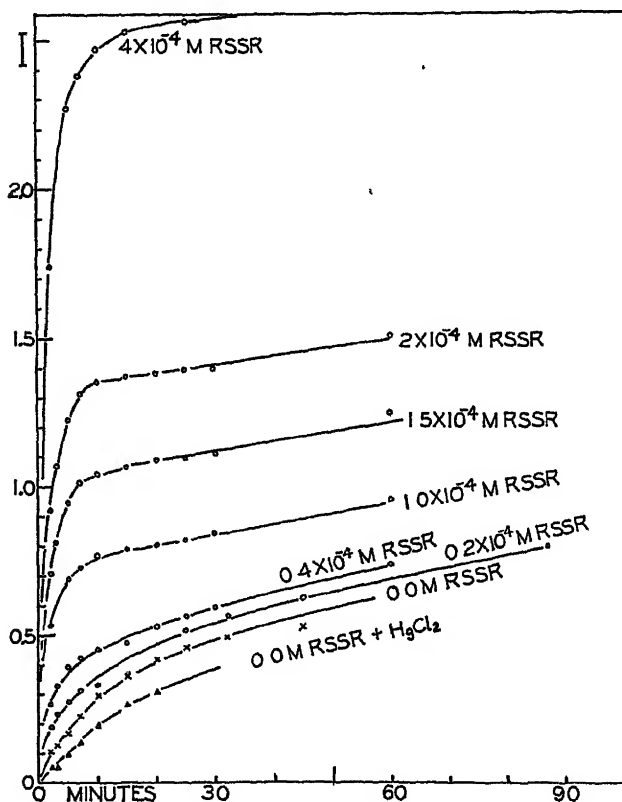


FIG. 3. Color production by cystine in urine with phospho-18-tungstic acid reagent in the presence of  $\text{NaHSO}_3$ . Composition of color solution, sodium acetate  $0.4\text{ M}$ , acetic acid  $0.1\text{ M}$ ,  $\text{NaHSO}_3$   $0.06\text{ M}$ , urine containing various amounts of cystine  $10\text{ cc}$  per  $50\text{ cc}$ , phospho-18-tungstic acid reagent  $4\text{ cc}$  per  $50\text{ cc}$ ; total volume  $50\text{ cc}$ . Color standard, solution containing  $4 \times 10^{-4}\text{ M}$  cystine and an adequate amount of brom-thymol blue solution besides acetate buffer and reagent. Blank, solution containing the urine to which no cystine is added, and  $2\text{ cc}$  of  $0.1\text{ M}$   $\text{HgCl}_2$ .  $I$ , color intensity.

was added to a buffer of  $0.4\text{ M}$  sodium acetate and  $0.1\text{ M}$  acetic acid, to which  $0.06\text{ M}$   $\text{NaHSO}_3$  had been added, and treated in

the same way as the pure cystine solution (6). The results are given in Fig. 3 which shows that in all cases the intensity gradually increases even after 30 minutes and no apparent maximum is realized. This is due to greater color production by extraneous reducers<sup>1</sup> in the presence of  $\text{NaHSO}_3$ . In fact, the oxidized urine produces rather intense color, and that to which  $\text{HgCl}_2$  is added (blank) slightly less intensity. The difference in the two intensities is, no doubt, due to cystine originally present. An analytically important fact is that the intensities obtained by subtracting those of the corresponding blanks from those directly observed are approximately proportional to the cystine concentration (added plus that originally present).

*Establishment of Routine Methods for Determination of Cysteine, Cystine, and Ascorbic Acid in Urine*—Taking the above experimental observations into consideration, together with those reported previously (1-6), we devised the following methods.

*Reagents*

2 M sodium acetate solution.

2 M acetic acid solution.

Phospho-18-tungstic acid reagent prepared, as reported in Paper I (1), by the Folin and Marenzi method (7), except that the addition of lithium salt is omitted

2 M  $\text{HCHO}$  solution. Diluted commercial 38 per cent formaldehyde solution (c.p. grade). Its strength is determined by the  $\text{H}_2\text{O}_2$  oxidation method if necessary.

0.1 M  $\text{HgCl}_2$  solution. A definite amount of  $\text{HgCl}_2$  (c.p. grade) is dissolved in water. Accuracy is not necessary unless cysteine hydrochloride is standardized

10 M  $\text{NaHSO}_3$ . A commercial preparation (c.p. grade) is dissolved in water to which 50 cc. of 1 M  $\text{NaOH}$  per liter of the resulting solution are added. It may be used for 1 month at least, if well preserved.

0.01 M cysteine in 0.2 N  $\text{HCl}$  (or  $\text{H}_2\text{SO}_4$ ). Previous to its use commercial cysteine hydrochloride should be standardized according to the method described in Paper IV (4). The solution can be used for at least a few days if preserved at  $0^\circ$ .

<sup>1</sup> Oxidized ascorbic acid was found not to produce color even with sulfite (6).

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Brom-thymol blue solution. Ordinary indicator solution. Its strength need not be determined.

Color standard. First, two standard solutions are made, one containing 10 drops of the brom-thymol blue solution per 50 cc besides  $4 \times 10^{-4}$  M cysteine, 0.4 M sodium acetate, 0.12 M acetic acid, and 4 cc. per 50 cc. of the reagent; and the other containing all the above substances except brom-thymol blue. The two solutions are mixed in an adequate ratio, so that the color of the mixture is about the same as that of the test solution which contains urine, when compared colorimetrically. This mixture is used as the standard. It can be used for a period of 6 hours at least.

### *Technique*

*Cysteine Determination (Method 1)*—A test solution is made with 10 cc. of acetate and 3 cc. of acetic acid solutions, 10 cc. of the urine to be tested, and 4 cc. of the reagent, the total volume being 50 cc. The blank is made in exactly the same manner except that 6 cc. of HCHO solution are added 2 minutes before the reagent. The color intensities are determined in about 15 minutes.

The intensity of the test solution is symbolized by  $I_t$  and that of the blank by  $I_b$ . Then the following equation gives the molar concentration of cysteine in the test solution, from which the amount of cysteine in the urine is calculated.

$$C_0 (I_t - I_b) = C_{R-SH} \quad (1)$$

where  $C_0$  is the molar cysteine concentration ( $4 \times 10^{-4}$ ) of the standard solution and  $C_{R-SH}$  that of the test solution.

*Cystine Determination (Method 2)*—A test solution is made containing 10 cc. of acetate and 2 cc. of acetic acid solutions, 3 cc. of  $\text{NaHSO}_3$  solution, 10 cc. of the urine to be tested, and 4 cc. of the reagent, the total volume being 50 cc. The blank is made exactly the same except that 2 cc. of  $\text{HgCl}_2$  solution are added 2 minutes before the reagent. The color intensities are determined in 20 minutes.

Represent the intensity of the test solution by  $I'$ , and that of the blank by  $I'_b$ . Then Equation 2 gives the combined molar concentration of cysteine and cystine in the test solution, from

which the amount of cystine in the urine is easily found by the use of Equation 1.

$$C_0 \frac{I'_t - I'_b}{2} = C_{R-SH} + C_{R-S-S-R} \quad (2)$$

where  $C_{R-SH}$  and  $C_{R-S-S-R}$  are the respective molar concentrations of cysteine and cystine in the test solution.

*Ascorbic Acid Determination*—The amount of ascorbic acid can be obtained according to Equation 3 from the value of  $I_b$  found in the cysteine determination, provided the amount of the other extraneous reducers is negligible, which has so far been found to be the case.

$$C_0 (I_b/2) = C_a \quad (3)$$

where  $C_a$  is the molar concentration of ascorbic acid in the blank. If a perceptible amount of extraneous reducers (like creatinine<sup>2</sup>) is present, which is ascertained by oxidizing the urine by vigorously bubbling air through a part of it for 2 to 3 hours, the  $I_b$  value obtained with it ( ${}_oI_b$ ) is subtracted from the original  $I_b$  value (obtained with fresh urine). The difference gives  $C_a$  according to Equation 3-a.

$$C_0 \frac{I_b - {}_oI_b}{2} = C_a \quad (3-a)$$

The  $I'_b$  value also gives the approximate value of  $C_a$ . However, this is complicated by the contribution of color by  $NaHSO_3$  and also by the other extraneous reducers in its presence.

*Test for Applicability of the Methods*—In order to demonstrate the accuracy of the methods, several series of determinations were carried out in a routine manner with oxidized urine to which the three substances had been added in various ratios. In a few cases, blank solutions with either  $HCHO$  or  $HgCl_2$  were observed to form precipitate. The results are presented in Table I. Here the amounts of the three substances added and found are expressed as their molarity in the test solutions. Figures for the cystine found represent the sum of the substances originally

<sup>2</sup> The color produced by creatinine of less than  $2 \times 10^3$  M in the test solution may be disregarded (1).



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TABLE I

*Recovery of Cysteine, Cystine, and Ascorbic Acid Added to Oxidized Urine*

Urine sample No	R-SH added	R-S-S-R added	Ascorbic acid added	$I_t$	$I_b$	$I_t - I_b$	$I'_t$	$I'_b$	$I'_t - I'_b$	R-SH found	R-S-S-R found	Ascorbic acid found
	$M \times 10^4$	$M \times 10^4$	$M \times 10^4$							$M \times 10^4$	$M \times 10^4$	$M \times 10^4$
1	0 0			0 061	0 061	0 000						
	0 4			0 135	0 069	0 066				0 264		
	1.2			0 314	0 085	0 229				0 916		
	2 0			0 518	0 070	0 448				1 792		
	3 0			0 818	0 071	0 747				2 988		
	4 0			0 987	0 070	0 917				3 668		
	6 0			1 580	0 073	1 507				6.028		
2	0 0						0 119	0 066	0.053	0 106		
	0 4						0 289	0 065	0 224	0 448		
	1 0						0 568	0 063	0 505	1 010		
	1 5						0 799	0.065	0 734	1 468		
	2 0						1 079	0 065	1 014	2 028		
	3 0						1 495	0 065	1 430	2 860		
	4 0						1 980	0 063	1.920	3 840		
3	0 0						0 250	0 150	0 100	0 200		
	0 4						0 413	0.115	0 298	0 596		
	2 0						1 102	0 116	0 986	1 972		
	4 0						2 088	0 116	1 872	3 774		
4	0 0	0 0					0 183	0 085	0 098		0 196	
	0 2						0 208	0 091	0 117		0.234	
	0 4						0 249	0 100	0 149		0 298	
	1 0						0 543	0 100	0 443		0.886	
	1 4						0 769	0 102	0 607		1 214	
	2 0						1 074	0 094	0 980		1.960	
	4 0						2 073	0 088	1 985		3.970	
5	0 0						0 299	0.108	0 191		0.382	
	0 4						0 385	0.109	0 276		0 552	
	1 0						0.627	0 113	0 514		1 028	
	2 0						1 053	0 105	0 948		1 896	
6	0 0	0 0		0.000	0 000	0 000	0.105	0 077	0 028		0 056	
	0 4	0 4		0 106	0 000	0 106	0 469	0 069	0 400	0 424	0 376	
	0 4	1 0		0 100	0 000	0 100	0 784	0 070	0 714	0 400	1 028	
	0 4	2 0		0 106	0 000	0 106	1 286	0 051	0 235	0 424	2 044	
	0 4	4 0		0 100	0 000	1 000	2 345	0 053	2 292	0 400	4 184	

TABLE I—*Concluded*

Urine sample No.	R-SH added	R-S-S-R added	Ascorbic acid added	$I_f$	$I_b$	$I_t - I_b$	$I'_f$	$I'_b$	$I'_t - I'_b$	R-SH found	R-S-S-R found	Ascorbic acid found
	$\mu \times 10^4$	$\mu \times 10^4$	$\mu \times 10^4$							$\mu \times 10^4$	$\mu \times 10^4$	$\mu \times 10^4$
7	0 0 0 0			0 000	0 000	0 000	0 083	0 045	0 038			
	1 0 0 4			0 251	0 000	0 251	0 745	0 046	0 699	1 004	0 394	
	1 0 1 0			0 249	0 000	0 249	1 076	0 047	1 029	0 996	1 064	
	1 0 2 0			0 249	0 000	0 249	1 622	0 047	1 575	0 996	2 153	
	1 0 4 0			0 248	0 000	0 248	2 597	0 046	2 551	0 992	4 110	
8	0 0 0 0			0 000	0 000	0 000	0 127	0 069	0 058			0 116
	2 0 0 2			0 499	0 000	0 499	1 153	0 066	1 087	1 996	0 180	
	2 0 0 4			0 499	0 000	0 499	1 270	0 065	1 205	1 996	0 416	
	2 0 1 0			0 498	0 000	0 498	1 571	0 065	1 506	1 992	1 020	
	2 0 2 0			0 493	0 000	0 493	2 121	0 062	2 059	1 972	2 146	
9	0 0 0 0			0 000	0 000	0 000	0 074	0 025	0 049			0 098
	4 0 0 2			0 998	0 000	0 998	2 069	0 032	2 037	3 992	0 084	
	4 0 0 4			1 000	0 000	1 000	2 143	0 033	2 110	4 000	0 220	
	4 0 1 0			1 003	0 000	1 003	2 473	0 033	2 440	4 012	0 868	
	4 0 2 0			1 000	0 000	1 000	2 752	0 032	2 720	4 000	1 440	
10			0 000	0 065	0 065	0 000				0 000		0 130
			0 000	0 089	0 088	0 001				0 004		0 176
			0 367	0 211	0 230	-0 020				-0 080		0 460
			0 917	0 499	0 498	0 001				0 004		0 996
			1 835	0 922	0 922	0 000				0 000		1 844
			3 670	1 860	1 843	0 017				0 068		3 686
			0 000				0 128	0 089	0 039		0 078	0 178
			0 367				0 284	0 240	0 044		0 088	0 480
			0 917				0 551	0 494	0 057		0 114	0 988
			1 835				0 962	0 903	0 059		0 118	1 806
11	0 0		0 000	0 040	0 048	-0 008				-0 032		0 096
	0 4		1 303	0 805	0 702	0 103				0 412		1 404
	2 0		2 606	1 794	1 365	0 429				1 716		2 730
	4 0		0 261	1 227	0 177	1 050				4 200		0 354

present in and added to urine. Therefore, the value of the cystine found for oxidized urine itself should be subtracted from them, if the cystine added and that recovered are to be compared. The values for ascorbic acid found also represent the sum of the ascorbic acid added and extraneous reducers present in the oxi-

dized urine and expressed in the molarity of ascorbic acid. Therefore the value of the "ascorbic acid found" of the oxidized urine itself should be subtracted from each for comparison with those added.

The cysteine found can be taken by itself, as the amount of cysteine in the oxidized urine is 0, within the limits of experimental error, which are seen under " $I_t - I_b$ ."

The results of the determinations indicate the following facts. (1)  $I_b$  values are the same within the limits of experimental error ( $\pm 0.01$ ) regardless of the cysteine concentration, as is seen under  $I_b$  for urine Sample 1. (2)  $\text{asc}I_b$  values of urine Samples 1, 10, and 11 are not 0 but 0.061, 0.065, and 0.048 respectively. This fact is due possibly to incomplete oxidation of urine, although partly to some extraneous reducers. (3)  $\text{asc}I_t - \text{asc}I_b$  ( $I_t - I_b$  of oxidized urine) values of each sample, and also  $I_t - I_b$  values of Sample 10, to which varying amounts of ascorbic acid only are added, are seen to be 0, within the limits of experimental error, which clearly indicates that the oxidized urines contain no cysteine. (4) The values for the cysteine found of Samples 1, 6, 7, 8, and 9 show the high accuracy of Method 1 in ordinary cases, although it apparently decreases with increase in ascorbic acid concentration, as seen in Sample 11. (5) As seen from the ascorbic acid found of Samples 10 and 11, ascorbic acid can accurately be determined by Method 1. (6)  $I'_b$  values for the same urine are the same within the limits of experimental error, regardless of cysteine or cystine concentrations, as seen for Samples 2 to 9. (7)  $I'_t - I'_b$  values of the oxidized urine are rather high, which clearly indicates the presence of cystine in it. As seen for Sample 10,  $I'_t - I'_b$  values are the same for the same urine regardless of the ascorbic acid concentration. (8) As seen for Samples 2 to 10, the values of the cystine and also cysteine found by Method 2, which are obtained by subtracting those of the oxidized urines from those of urines to which cysteine or cystine has been added, are smaller than the amounts added. This fact suggests that the color development is not complete within the time limits. However, the values may be considered as satisfactory for biological materials. (9) As seen for urine Samples 5 to 9,  $\text{asc}I'_b$  values are far greater than  $\text{asc}I_b$  values of the same

\*  $I'_b$  represents the intensity of oxidized urine in the presence of  $\text{HgCl}_2$  and  $\text{NaHSO}_3$ , while  $\text{asc}I_b$  is the intensity in the presence of  $\text{HCHO}$  and without  $\text{NaHSO}_3$ .

urine samples, since  $\text{NaHSO}_3$  contributes some color, besides that produced by extraneous reducers in its presence.

In general, the methods may be considered quite satisfactory for the differential determination of the three substances in urine

TABLE II  
*Determination of Cysteine, Cystine, and Ascorbic Acid in Fresh Normal Urine*

	Time of urine collection	Cysteine	Cystine	Ascorbic acid
		mg per 100 cc	mg per 100 cc	mg per 100 cc
Subject A	1st day, 10 30 a.m.	0 07	2 5	3 2
	2 00 p.m. *	0 5	1 6	11 25
	4 45 "	0 35	1 1	6 0
Subject B	2nd day, 9 30 a.m.	0 6	2 6	5 7
	1st day, 12 30 p.m.	0 85	2 7	5 35
	3 00 " *	0 8	4 8	11 6
Subject C	1st day, 10 45 a.m.	0 0	3 8	17 9
	1 15 p.m. *	0 3	3 6	26 8
	4 20 "	0 5	2 0	22 1
	2nd day, 9 30 a.m.	0 6	2 8	5 4
Values found by Virtue and Lewis (8)			1 1- 7 5	
Looney (9)			11 8-12 5	
Van Eekelen <i>et al</i> (10)			0- 10 0	
Harris <i>et al</i> (11)			4 0 (Average)	
				15 0 -25 0
				0 75-25 0
				2 0 - 4 0
				(Normal)

\* The urine was collected 2 to 3 hours after taking about 150 cc of orange juice.

*Determination of Cysteine, Cystine, and Ascorbic Acid in Fresh Urine*—After the methods had been found reliable, they were applied to the analysis of fresh urines from normal persons. The determinations were made as soon as the samples were collected, their results being shown in Table II.

As seen in Table II, the amounts of cysteine found are all very small. However, the maximum deviation which takes place in cysteine determination is less than 0.2 mg. according to Table I,

while the values here found are generally 3 to 4 times greater. For this reason the values may be taken as representing the amount actually present in urine. It is naturally expected that there is more or less cysteine in urine, which is a reducing system.

The amount of cystine found is rather constant, ranging from 1.1 to 4.8 mg. per 100 cc. Without using the same urine, comparison with the values found by other workers cannot be made; however, the agreement, at least in the order of magnitude with these values, proves the applicability of the method. The amounts of ascorbic acid found here are also in good agreement with those previously found by other workers (10, 11) by different methods.

To test the actual presence of cysteine or cystine in the urine, excess  $\text{HgCl}_2$  solution was added to fresh urine and the precipitate was separated and washed many times with water and acetone. It was found to contain sulfur. The precipitate was decomposed by  $\text{H}_2\text{S}$ , the  $\text{HgS}$  precipitate was filtered off, and the  $\text{H}_2\text{S}$  in the filtrate driven off. The final solution gave a distinct red color in the modified nitroprusside test (12) and roughly equivalent color was produced in the modified phospho-18-tungstic acid test. The filtrate from which  $\text{HgS}$  precipitate is filtered off does not give color in the modified phospho-18-tungstic acid test. The filtrate obtained by precipitating  $\text{Hg}^{++}$  as  $\text{HgS}$  by  $\text{H}_2\text{S}$  and driving off the dissolved  $\text{H}_2\text{S}$  with nitrogen again produces color in the modified phospho-18-tungstic acid test, but no color in the modified nitroprusside test.

These tests are qualitatively in accord with the findings by the modified phospho-18-tungstic acid methods.

In a strict sense, cysteine and cystine may possibly be reduced and oxidized glutathione. However, associated with cystinuria, they may be accepted as they are, since there is at present no method which separates the amino acids from their tripeptides.

To establish normal cysteine and cystine contents, more extensive work should be carried out to obtain average values.  $\text{HCHO}$  used in the blank may be substituted by iodoacetic acid, possibly with more advantage. The use of  $\text{HgCl}_2$  may successfully accomplish the separation and more accurate determination of the sulfur compounds from ascorbic acid and other reducers. However, these aspects are left to future study.

## SUMMARY

The phospho-18-tungstic acid methods for differential determinations of cysteine, cystine, and ascorbic acid were tested for their applicability to urinalysis. Good recovery of the three substances added to oxidized urine was obtained.

By these methods, the presence of 0.0 to 0.9 mg. of cysteine per 100 cc of fresh urine, which had been overlooked by previous workers, and 1.1 to 4.8 mg. of cystine and 3.2 to 26.8 mg. of ascorbic acid per 100 cc. of fresh normal urine were found.

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## THE CARBAMATE EQUILIBRIUM\*

### I. THE EQUILIBRIUM OF AMINO ACIDS, CARBON DIOXIDE, AND CARBAMATES IN AQUEOUS SOLUTION; WITH A NOTE ON THE FERGUSON-ROUGHTON CARBAMATE METHOD

By WILLIAM C STADIE AND HELEN O'BRIEN†

(From the John Herr Musser Department of Research Medicine, University of Pennsylvania, Philadelphia)

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#### Notation

$$\begin{aligned}
 (N) &= \text{total amino acid, mm per liter} \\
 (Z^\pm) &= \begin{array}{c} \text{NH}_3^+ \\ | \\ \text{R} \\ | \\ \text{COO}^- \end{array} = \text{amino acid zwitter ion, mm per liter} \\
 (Z^-) &= \begin{array}{c} \text{NH}_2 \\ | \\ \text{R} \\ | \\ \text{COO}^- \\ | \\ \text{NH} \cdot \text{COO}^- \end{array} = \text{ " " amphamion " " " } \\
 (\text{Am}^-) &= \begin{array}{c} \text{COO}^- \\ | \\ \text{R} \\ | \\ \text{COO}^- \end{array} = \text{ " " carbamate " " " } \\
 (\text{BHCO}_3^-) &= \text{bicarbonate, mm per liter} \\
 (\text{B}_2\text{CO}_3^-) &= \text{carbonate, mm per liter} \\
 \alpha_{\text{CO}_2} P_{\text{CO}_2} &= \text{physically dissolved CO}_2 \\
 (\text{B}_{\text{On}}) &= (\text{BHCO}_3^-) + 2(\text{B}_2\text{CO}_3^-) \\
 &= \alpha_{\text{CO}_2} P_{\text{CO}_2} \frac{K_1}{(\text{H}^+)} \left( 1 + \frac{2K_2}{(\text{H}^+)} \right) \quad (1) \\
 &= \text{base as carbonates, m -eq. per liter} \\
 (\text{On}) &= (\text{BHCO}_3^-) + (\text{B}_2\text{CO}_3^-)
 \end{aligned}$$

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† J Allison Scott Fellow.



$$= \alpha_{\text{CO}_2} P_{\text{CO}_2} \frac{K_1}{(\text{H}^+)} \left[ 1 + \frac{K_2}{(\text{H}^+)} \right] \quad (2)$$

= CO<sub>2</sub> as carbonates, mm per liter

(B<sup>+</sup>) = available base, m.-eq. per liter

(H<sup>+</sup>) = hydrogen ion activity

K<sub>1</sub> = so called first acid dissociation constant of H<sub>2</sub>CO<sub>3</sub> (Equations 1 and 2)

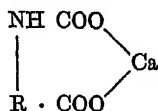
K<sub>2</sub> = second dissociation constant of H<sub>2</sub>CO<sub>3</sub> (Equations 1 and 2)

K<sub>Z</sub> = dissociation constant of amino acid (Equations 3 and 4)

K<sub>Am</sub> = mass action constant of amino acid-carbamate equilibrium (Equation 5)

### INTRODUCTION

That CO<sub>2</sub> combines with ammonia, amines, and amino acids to form carbamates was early recognized and studied in detail by Siegfried (1908) and by Faurholt (1925). In the case of ammonia the reaction has usually been written  $\text{NH}_3 + \text{CO}_2 = \text{NH}_2 \cdot \text{COOH}$ . The resulting carbamic acid has been isolated in the form of its salts and esters. Analogous compounds of amino acids are formed by the action of CO<sub>2</sub>, *viz.*  $\text{COOH} \cdot \text{R} \cdot \text{NH}_2 + \text{CO}_2 = \text{COOH} \cdot \text{R} \cdot \text{NH} \cdot \text{COOH}$ . They have been isolated as crystalline calcium salts by Siegfried. According to him the carbamates have the general formula corresponding to a dibasic salt, *viz.*



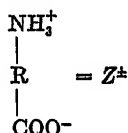
Recently the notion that forms of CO<sub>2</sub> other than bicarbonate are present in blood and are important as carriers of CO<sub>2</sub> has stimulated a renewed interest in the chemistry of the carbamates. In brief, the original idea of Bohr (1905), that CO<sub>2</sub> combines directly with hemoglobin in a manner analogous to its combination with oxygen, has been replaced by the conception that CO<sub>2</sub> combines with one or more amino groups of the protein to form hemoglobin carbamates, *viz.*  $\text{HbNH}_2 + \text{CO}_2 = \text{HbNH} \cdot \text{COO}^- + \text{H}^+$ . This aspect of the subject has recently been well reviewed by Roughton (1935).

In this paper we have sought to do two things: (1) we have applied the mass action law to the equilibria of amino acids and

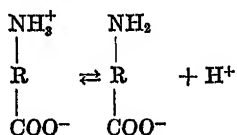
CO<sub>2</sub> and have derived equations which describe these equilibria under a variety of conditions; (2) we have experimentally determined these equilibria and have tested the validity of the mass law equations by these data. The theory is satisfactorily supported by the facts and we believe it to be the first adequate treatment of the amino acid-CO<sub>2</sub> equilibrium. We have already shown (Stadie and O'Brien, 1935) and in a separate paper will discuss in detail how the theory will account for the known experimental facts with respect to the carbamate equilibrium in the case of hemoglobin and serum albumin

### *Ionization of the Amino Acid*

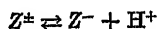
The coexistence upon the same molecule of an amino and a carboxyl group has led to the currently accepted view, first presented by Bjerrum, that amino acids exist in solution at the isoelectric pH<sup>+</sup> almost entirely as zwitter ions,<sup>1</sup> viz.



The ionization of the zwitter ions on the alkaline side of the isoelectric point may be represented by the equation



or



This equilibrium is represented by the equation

$$\frac{(\text{Z}^-)(\text{H}^+)}{(\text{Z}^\pm)} = K_z \quad (3)$$

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<sup>1</sup> For the evidence supporting the zwitter ion hypothesis see Sherwin and Harrow (1935).

which may be transformed into the convenient "salt" form

$$(Z^-) = \frac{K_z}{K_z + (H^+)} [(Z^-) + (Z^\pm)] \quad (4)$$

The addition of base to isoelectric amino acid increases  $pH^+$  and simultaneously increases the proportion of the acid existing as  $Z^-$ . When 1 equivalent of base per mole of amino acid is added, all of the amino acid exists as  $Z^-$ . For amounts of base less than 1 equivalent the proportion of acid as  $Z^-$  is given by Equation 4. So far as it is known, this variation of the relative amounts of  $Z^-$  and  $Z^\pm$  is the only effect of the variation of  $(H^+)$  in an amino acid solution.

#### *Combination of $CO_2$ with Amino Acid*

Either in the literature or as reported here the combination of  $CO_2$  with an amino acid has been shown by experiment to have the following characteristics.

1. The preparation and isolation of calcium salts of carbamate can only be accomplished in a very alkaline medium (Siegfried, 1908).

2. The proportion of amino acid converted into carbamate by combination with  $CO_2$  is greater the higher the  $pH^+$  (Faurholt, 1924; Meldrum and Roughton, 1933).

3. No combination of  $CO_2$  as carbamate can be demonstrated with isoelectric amino acid. We show this by the following experiment.

*Isoelectric Glycine (Zwitter Ion)- $CO_2$  Equilibrium*—Isoelectric glycine, 0.1 M, was equilibrated with  $CO_2$  in tonometers for periods of 4 to 6 hours. The solution and the gas phases were then separated and analyzed. The results of this experiment are shown in Table I. The carbamate calculated from the total  $CO_2$  and  $\alpha_{CO_2}P_{CO_2}$  is within the limits of error, negligibly small. In addition the observed carbamate (the  $CO_2$  not precipitated by barium) is but 0.3 mm per liter, an amount no greater than that observed in control determinations in which the presence of carbamate is excluded. Further, the amount is constant despite an almost 10-fold increase in  $P_{CO_2}$ .

It is evident from this experiment that  $CO_2$  does not combine

with isoelectric glycine (zwitter ion), nor, by presumption, with other amino acids, as carbamate.

TABLE I

*Equilibrium of Isoelectric Glycine and CO<sub>2</sub> (No Available Base Present)*

Glycine = 0.1 M per liter.

$\alpha\text{CO}_2 = 4.87 \times 10^{-2}$  mm per liter per mm. of Hg;  $t = 22^\circ$ .

The observed carbamate was determined by the Ferguson-Roughton method in the presence of carbonic anhydrase. Cf. text

Experiment No	$P_{\text{CO}_2}$	pH <sup>+</sup>	Total CO <sub>2</sub>	$\alpha\text{CO}_2 P_{\text{CO}_2}$	Carbamate	
					Calculated by difference	Observed
	mm Hg		mm per l	mm per l	mm per l	mm per l
1	86	5.22	4.8	4.5	0.3	0.4
2	167	4.88	9.5	8.6	0.9	0.2
3	264	4.74	13.4	13.6	-0.2	0.0
4	340	4.64	18.5	17.4	1.1	0.4
5	732	4.54	32.8	35.7	-2.9	0.3
Mean	.	..	.	.	.	0.3

TABLE II

*Equilibrium of Isoelectric Glycine and CO<sub>2</sub> in Presence of Bicarbonate*

Original solution

Glycine = 0.1 M per liter

$t = 22^\circ$

NaOH = 0.053 M per liter

$\alpha\text{CO}_2 = 0.0489$  mm per liter per mm. Hg

Equilibrated solution

$P_{\text{CO}_2}$ . . . . .	732	mm. Hg
pH <sup>+</sup> (observed) . . . . .	6.50	
" (calculated) . . . . .	6.47	
Free CO <sub>2</sub> . . . . .	35.7	mm per liter
Base as $\text{BHCO}_3^-$ . . . . .	53.0	" " "
Sum. . . . .	88.7	" " "
Total CO <sub>2</sub> (observed) . . . . .	87.7	" " "
Difference . . . . .	-1.0	" " "
Carbamate* (observed) . . . . .	0.1	" " "

\* Determined by the modified Ferguson-Roughton method

4. The bicarbonate or carbonate ion does not combine with amino acid. This is shown by the two following experiments.

*When the Amino Acid Is All Zwitter Ion*—A solution of glycine containing base was equilibrated for 12 hours with pure  $\text{CO}_2$ . The  $\text{pH}^+$  (6.50) was sufficiently acid to assume that all glycine was zwitter ion and all base bicarbonate. Analysis showed the results presented in Table II.

The experiment shows conclusively that the free  $\text{CO}_2$  together with all the base as bicarbonate fully accounts for the total  $\text{CO}_2$ . No  $\text{CO}_2$  as carbamate need be accounted for. Further, only 0.1 mm per liter as carbamate was found directly by our modification of the Ferguson-Roughton method. Lastly, it might be noted that the  $\text{pH}^+$  calculated by the equation

$$\text{pH}^+ = 6.30 + \log (\text{NaHCO}_3^- / \alpha_{\text{CO}_2} P_{\text{CO}_2})$$

agrees closely with the observed values. This is further evidence for the conclusion that at  $\text{pH}^+$  where all amino acid exists as zwitter ion no combination of the amino acid with free  $\text{CO}_2$  or bicarbonate occurs.

*When the Amino Acid Is Part Amphion*—The velocity of carbamate formation depends upon the concentration of free  $\text{CO}_2$ . It would be expected (if the concentration of free  $\text{CO}_2$  were very small) that the time required for equilibrium to be established would be great, even in the presence of amphanion and  $\text{HCO}_3^-$  or  $\text{CO}_3^{--}$ . This is found to be so, as shown by the following experiment

A 0.05 M glycine solution was quickly mixed with 0.25 M  $\text{Na}_2\text{CO}_3$  solution and the  $\text{pH}^+$  of the mixed solution determined by means of the glass electrode over a period of 24 hours. The results are shown in Fig. 1.

It should be noted that at 0 time the calculated composition of the solution shows a considerable portion of glycine as anion, appreciable amounts of  $\text{HCO}_3^-$  and  $\text{CO}_3^{--}$ , and a minute concentration of free  $\text{CO}_2$ .

As expected, the velocity of the reaction, as indicated by the rate of change of  $\text{pH}^+$ , is very slow, in sharp contrast to the rapidity with which equilibrium is reached when appreciable concentrations of free  $\text{CO}_2$  are present (see Fig. 4).

From this experiment we conclude that neither  $\text{HCO}_3^-$  or  $\text{CO}_3^{--}$  *per se*, but only free  $\text{CO}_2$ , reacts with glycine, even when it exists as amphion.

5. Carbamate formation is inhibited in the presence of formol. As is well known, formalin reacts with free amino groups to form methylene compounds. It should be expected that in the presence of formalin the ability of amino acids to react with  $\text{CO}_2$  to form carbamates would be partly or wholly lost. That this is the case is seen from the following experiments.

A solution of 0.1 M glycine with 0.1 M NaOH with and without formol was equilibrated with  $\text{CO}_2$  at  $0^\circ$ . The concentration of carbamate when equilibrium had been established in the absence of carbonates, was found to be 46 mM per liter without formol and

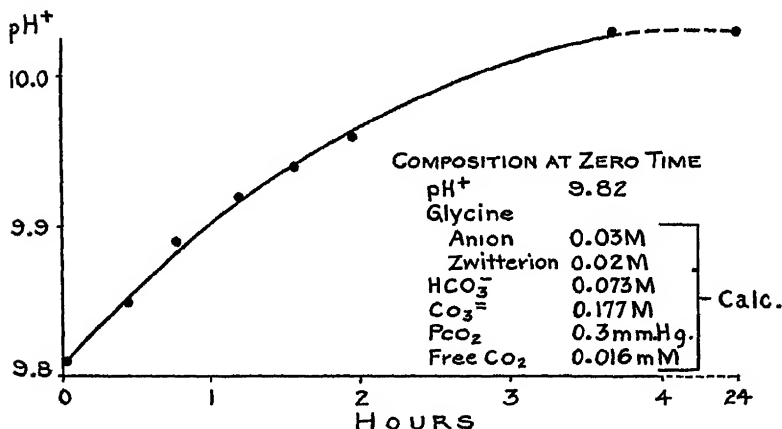


FIG. 1 Velocity of carbamate formation in a glycine solution at alkaline pH<sup>+</sup> in the presence of  $\text{HCO}_3^-$  and  $\text{CO}_3^{=}$  (free  $\text{CO}_2$  very small) Composition at 0 time (except pH<sup>+</sup>) calculated.

17<sup>2</sup> mM per liter with 0.1 to 0.6 M per liter of formol. The experiment shows that in the presence of formol the amount of carbamate formed is considerably less than in the control. This is further evidence that  $\text{CO}_2$  combines with the amino group of the amino acid.

*First Assumption for the Carbamate Equilibrium*—These five characteristics of the  $\text{CO}_2$ -amino acid combination enable us to state our first assumption for the development of the mass action equations of the equilibrium of amino acids and  $\text{CO}_2$ .

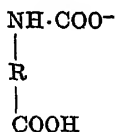
<sup>2</sup> Mean of four concordant determinations.

Free  $\text{CO}_2$  only reacts with amphoteric,  $\text{COO}^- \cdot \text{R} \cdot \text{NH}_2$ , and not with the zwitter ion,  $\text{COO}^- \cdot \text{R} \cdot \text{NH}_3^+$ , of the amino acid. This assumption was first made by Faurholt and concurred in by Meldrum and Roughton. It will be found subsequently to be in complete accord with the experiments reported in this paper.

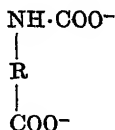
*Change of ( $\text{H}^+$ ) Attendant upon the Combination of  $\text{CO}_2$  and Amino Acid*

*Second Assumption for the Carbamate Equilibrium*—When  $\text{CO}_2$  reacts with the amphoteric of an amino acid, viz.  $\text{COO}^- \cdot \text{R} \cdot \text{NH}_2 + \text{CO}_2$ , there is no *a priori* reasoning which can predict with certainty the behavior of the resultant carbamino compound with respect to hydrogen ions. The carbamino compound might, for each mole of  $\text{CO}_2$  bound, (a) ionize 1 equivalent of  $\text{H}^+$ , (b) ionize a fraction of an equivalent of  $\text{H}^+$ , (c) ionize no additional  $\text{H}^+$ , (d) combine with 1 or less equivalent of  $\text{H}^+$ .

In point of fact it is found that (a) is the only alternative which corresponds to the experiments designed to test these possibilities. It must be further decided, moreover, whether the carbamino compound formed has a single negative charge corresponding to the formula



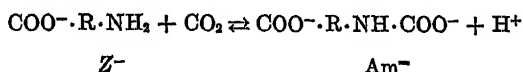
or a double negative charge corresponding to



It is found that the second of these alternatives is in conformity with our experiments. This leads at once to our second assumption: *the carbamino  $\text{CO}_2$  compounds of amino acids are dibasic salts at all  $\text{pH}^+$  values  $> ca. 7$ .* This is equivalent to saying that these carbamic acids are dibasic acids whose acid dissociation constants are both equal to or more than  $ca. 10^{-6}$ . The proof of our second assumption will be given below.

*The Carbamate Mass Action Constant*

In accordance with the two assumptions stated above and which are the only ones necessary for the complete development of the mass action equations for the equilibrium of  $\text{CO}_2$  and amino acids, we may now write the equation for the reaction between an amino acid and  $\text{CO}_2$ , viz.



For this reaction the mass action equation is

$$\frac{(\text{Am}^-)(\text{H}^+)}{(\text{Z}^-)\alpha_{\text{CO}_2}P_{\text{CO}_2}} = K_{\text{Am}} \quad (5)$$

It is our purpose to show that this mass action constant  $K_{\text{Am}}$  governs the equilibrium of amino acids and  $\text{CO}_2$  under a variety of conditions. By suitable alterations Equation 5 can be shown to be in good agreement with experiment. In a subsequent paper we propose to show that a similar equation is in conformity with the carbamate- $\text{CO}_2$  equilibrium in the case of proteins such as hemoglobin and horse serum proteins.

*Base Relations in the Amino Acid- $\text{CO}_2$  Equilibrium*

As was stated above,  $\text{CO}_2$  does not combine with the isoelectric amino acid. Available base must be added in an amount equal to 1 equivalent per mole or less of acid in order for combination to occur.<sup>3</sup> At equilibrium ( $\text{pH} \leq 12$ ,  $\text{OH}^- \leq 0.001 \text{ M}$ ) the base is expressed by Equation 6.

$$(\text{B}^+) = (\text{Z}^-) + 2(\text{Am}^-) + (\text{BHCO}_3^-) + 2(\text{B}_2\text{CO}_3^{--}) \quad (6)$$

It will be shown below that under special conditions it is possible to establish an equilibrium between amino acid and  $\text{CO}_2$  in which no bicarbonate or carbonate is formed. In that case, the base is expressed by

$$(\text{B}^+) = (\text{Z}^-) + 2(\text{Am}^-) \quad (7)$$

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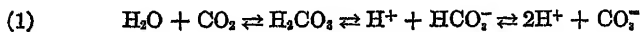
<sup>3</sup> The case in which the equivalents of base added are in excess of 1 will not be discussed. This case, however, may easily be treated by an extension of the methods used here.



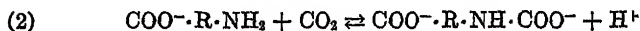
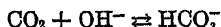
These two equations, *i.e.* Equations 5 and 6 (or Equation 7), are merely the mathematical expressions for the two assumptions outlined above.

*Two Types of Equilibria Possible in Reaction between an Amino Acid and CO<sub>2</sub>*

*Equilibrium in Absence of H<sub>2</sub>CO<sub>3</sub> or Its Ions HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>=</sup>; or "Non-Carbonate" Equilibrium*—An aqueous amino acid solution which contains 1 equivalent or less of base, and is therefore at a pH<sup>+</sup> greater than its isoelectric point, when equilibrated with a gas phase containing CO<sub>2</sub> reacts, so far as we know, in two ways only with the CO<sub>2</sub>, *viz.*



or



Reaction (1), the hydration of CO<sub>2</sub> to form carbonic acid, is a slow one, particularly at low temperatures, as has been amply demonstrated by Faurholt (1924), Brinkman, Margaria, and Roughton (1933), and Stadie and O'Brien (1933).

Reaction (2), the formation of carbamates, on the other hand, is rapid even at 0° (Faurholt, 1925). Assume a solution of an amino acid to be rapidly equilibrated with a CO<sub>2</sub> gas phase for a short time only. It is possible to suppose that the formation of carbamate would be practically completed, whereas the carbonic acid formation will be scarcely initiated. In effect, there would be an equilibrium established which would be one involving amino acid, carbamate, and CO<sub>2</sub> but in which carbonic acid and its ions HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>=</sup> are virtually absent.

We will show subsequently that this type of equilibrium is experimentally realizable. This "non-carbonate" equilibrium, as we have chosen to call it, has considerable importance. It brings out certain relations of the carbamate-CO<sub>2</sub> equilibrium which would otherwise be obscure. Further, its mathematical treatment is simpler and leads logically to a consideration of the second equilibrium in which carbonates must also be considered.

*Equilibrium in Presence of H<sub>2</sub>CO<sub>3</sub> or "Total" Equilibrium*—If

the equilibration of the amino acid and  $\text{CO}_2$  is allowed to proceed for a sufficient time, the hydration of  $\text{CO}_2$  to form  $\text{H}_2\text{CO}_3$  and its ions will go on to completion. The equilibrium will now include  $\text{H}_2\text{CO}_3$ ,  $\text{HCO}_3^-$ , and  $\text{CO}_3^{2-}$  in addition to the carbamate and will be quite different from the first or non-carbonate equilibrium. We call this the "total" equilibrium

In the subsequent development it will become apparent that both types of equilibrium can be treated mathematically on the basis of the same assumptions. The equations expressing the equilibria are derived by completely analogous methods. Moreover, the method of approach employed in the case of amino acids can be applied with very little alteration to the case of hemoglobin, leading to a consistent and logical explanation of the experimental facts as we know them.

It must be emphasized that both the non-carbonate and the total equilibrium are true equilibria in the thermodynamic sense and therefore both can be treated by means of the mass action law.

*Mass Action Equation for the Non-Carbonate Equilibrium of Amino Acid and  $\text{CO}_2$  and Proof of the Second Assumption*

That part of the amino acid which combines with  $\text{CO}_2$  must no longer be regarded as amino acid *per se* but as carbamate, designated ( $\text{Am}^-$ ). The remainder exists either as the amphanion or the zwitter ion in amounts depending on the  $\text{pH}^+$ . Accordingly, the total amino acid may be divided into three parts,  $Z^-$  the amphanion,  $Z^\pm$  the zwitter ion, and  $\text{Am}^-$  the carbamate.

We express this

$$(N) = (Z^-) + (Z^\pm) + (\text{Am}^-) \quad (8)$$

But by Equations 5 and 3

$$(Z^-) = \frac{(\text{Am}^-)(\text{H}^+)}{\alpha_{\text{CO}_2} P_{\text{CO}_2} K_{\text{Am}}}$$

and

$$(Z^\pm) = \frac{(\text{H}^+)(Z^-)}{K_z} = \frac{(\text{H}^+)^2(\text{Am}^-)}{\alpha P_{\text{CO}_2} K_{\text{Am}} K_z}$$

Then by combination of these equations we get

$$(\text{Am}^-) = \frac{(N)}{\left[ 1 + \frac{(\text{H}^+)}{\alpha_{\text{CO}_2} P_{\text{CO}_2} K_{\text{Am}}} \left( 1 + \frac{(\text{H}^+)}{K_z} \right) \right]} \quad (9)$$

This equation, which involves our first assumption only, is applicable to both the non-carbonate and the total equilibria. It can be readily tested for its validity provided there can be measured simultaneously the  $\text{pH}^+$ ,  $P_{\text{CO}_2}$ , and  $(\text{Am}^-)$  for a series of equilibria. This we have successfully done in the case of the total equilibrium, as we shall show. In the case of the non-carbonate equilibrium we have not devised a technique for the simultaneous measurement of  $\text{pH}^+$ ,  $P_{\text{CO}_2}$ , and  $(\text{Am}^-)$ . We have, however, measured the pairs  $\text{pH}^+$ ,  $(\text{Am}^-)$  and  $P_{\text{CO}_2}$ ,  $(\text{Am}^-)$  in separate experiments. From Equations 7, 8, and 3 we obtain

$$(\text{H}^+) = K_z \left[ \frac{(N) - (\text{B}^+) + (\text{Am}^-)}{(\text{B}^+) - 2(\text{Am}^-)} \right] \quad (10)$$

This equation, which we will prove by experiment, gives  $(\text{H}^+)$  in the case of the non-carbonate equilibrium when the concentration of carbamate is determined and when the constant  $K_z$  and the primary composition  $(\text{B}^+)$  and  $(N)$  are known. Equation 10 eliminates  $(\text{H}^+)$  from Equation 9 and allows  $(\text{Am}^-)$  to be expressed as a function of  $P_{\text{CO}_2}$  and the constants  $(\text{B}^+)$ ,  $(N)$ ,  $K_z$ .

The proof of this equation, which becomes the proof of our second assumption, is contained in the following experiments.

A 0.1 M glycine solution, to which had been added 0.05 M NaOH and 0.1 M NaCl, was placed in a bubbling hydrogen electrode, arranged in the conventional way for the determination of  $\text{pH}^+$ . Hydrogen was bubbled through the solution until constant readings of the E.M.F. were obtained. Then a 50 per cent mixture of  $\text{CO}_2$  and  $\text{H}_2$  was bubbled through the solution for periods varying from 1 to 60 seconds, whereupon the E.M.F. was again measured within 5 to 10 seconds from the cessation of the bubbling of  $\text{CO}_2$  gas. A sample of the solution was then removed for analysis by the Van Slyke method for  $\text{CO}_2$ . The  $\text{pH}^+$  change was measured a few seconds after equilibration, during which time the hydration of  $\text{CO}_2$  was certainly very small. In other words, the equilibrium involved carbamate only. All the  $\text{CO}_2$  is carbamate and Equation 10 is applicable.

The results of the experiments at  $0^\circ$  and  $15^\circ$  are shown in Fig. 2. The full line in both cases was calculated by Equation 10. The

value of  $pK_z$  used was calculated from the  $pH^+$  of the solution in the absence of  $CO_2$  by the Henderson-Hasselbalch equation.

$$pK_z = pH^+ - \log \frac{(\text{Na glycinate})}{(\text{glycine})}$$

The close agreement of the observed points with the calculated values leaves no doubt that the second assumption as expressed by

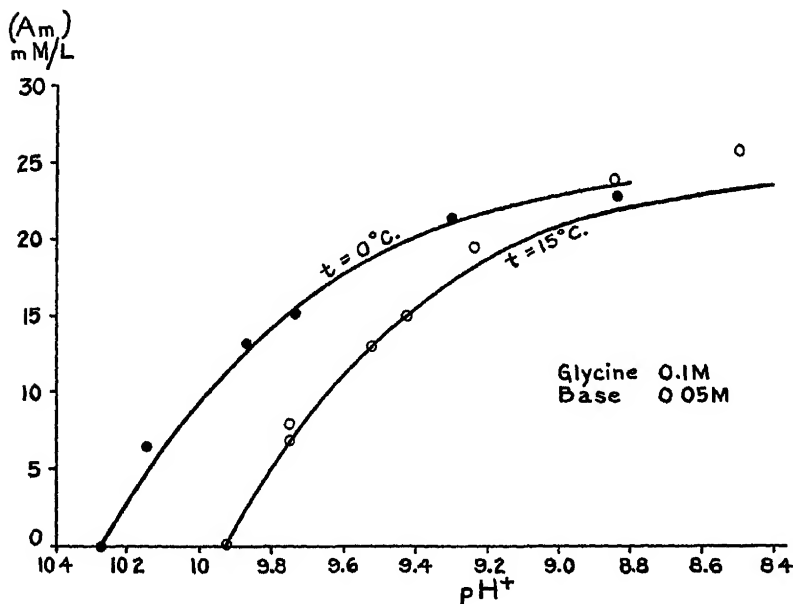


FIG. 2. Comparison of the observed  $pH^+$  in the non-carbonate equilibrium of glycine with the value calculated by Equation 10.

Equation 10 is true. It can easily be shown that any of the other possibilities discussed in the section above, "Change of  $(H^+)$  attendant upon the combination of  $CO_2$  and amino acid," would completely fail to fit the data. Both these experiments show further that the maximum carbamate formed in the non-carbonate equilibrium is equal to half the available base, *i.e.* 25 mM per liter.

A more limited proof of our second assumption in the case of the non-carbonate equilibrium follows from a consideration of Equa-

tion 7. When in the limit ( $\text{Am}^-$ ) is increased ( $Z^-$ ) becomes zero. We should then expect to find, when an amino acid is equilibrated with increasing pressures of  $\text{CO}_2$  (no  $\text{H}_2\text{CO}_3$  forming), that  $(\text{Am}^-)_{\text{maximum}} = \frac{1}{2} (\text{B}^+)$ .

The proof of this equation was obtained in the following way. Solutions of amino acids containing varying proportions of base were equilibrated with  $\text{CO}_2$ . The equilibration was performed in the fashion described under "Method for measurement of the non-

TABLE III  
*Maximum Carbamate As  $P_{\text{CO}_2}$  Is Increased in 0.1 M Glycine; No Carbonate Present ( $t = 20^\circ$ )*

Experi- ment No	Base	$P_{\text{CO}_2}$	Carbamate Base	Experi- ment No	Base	$P_{\text{CO}_2}$	Carbamate Base
	m-eq per l	mm Hg			m-eq per l	mm Hg	
1	100	13	0.12	1	80	32	0.39
2	100	31	0.36	2	80	84	0.33
3	100	55	0.40	3	80	109	0.41
4	100	74	0.43	4	80	176	0.44
5	100	99	0.43				
6	100	125	0.42				
7	100	171	0.42				
1	60	21	0.33	1	40	14	0.31
2	60	36	0.42	2	40	24	0.30
3	60	37	0.40	3	40	39	0.44
4	60	38	0.37	4	40	88	0.46
5	60	60	0.41	5	40	291	0.48
6	60	84	0.44				
7	60	169	0.50				

carbonate equilibrium," following, so that the formation of  $\text{H}_2\text{CO}_3$  or its ions was virtually excluded. The carbamate concentration under these circumstances was measured with increasing values of  $P_{\text{CO}_2}$ . It will be observed (Table III) that in all cases the value of the ratio ( $\text{Am}^-$ ):(base) approaches a maximum which shows no tendency to increase as the  $P_{\text{CO}_2}$  is increased. In no cases does the maximum ratio exceed 0.5 nor does it differ beyond experimental limits from the value of 0.5 demanded by the hypothesis.

The validity of our second assumption having been established,

it is now possible to combine Equations 9 and 10 into one equation. We eliminate  $H^+$  and obtain

$$\frac{(Am^-) [(N) - (B^+) + (Am^-)]}{[(B^+) - 2(Am^-)]^2} = \alpha_{CO_2} P_{CO_2} \frac{K_{Am}}{K_z} \quad (11)$$

This is the mass action equation for the *non-carbonate equilibrium* of amino acid and  $CO_2$  in terms of stoichiometrical constants ( $N$ ) and  $(B^+)$ , the ionization constant  $K_z$ , and the determinable variables  $(Am^-)$  and  $P_{CO_2}$ .

#### *Method for Measurement of the Non-Carbonate Equilibrium*

By Equation 11 and a simultaneous determination of the concentration of carbamate and the partial pressure of  $CO_2$  at equilibrium, we may calculate the mass action  $K_{Am}$  for any given solution of an amino acid when the constants ( $N$ ),  $(B^+) \alpha_{CO_2}$ , and  $K_z$  are known. The method for the study of the amino acid- $CO_2$  equilibrium in the absence of carbonates is similar to the method first used by Meldrum and Roughton (1933) and described by them as the "boat" method. We, however, have used a specially constructed Van Slyke type equilibrating device, the assembly of which is shown in Fig. 3.

The special features of the apparatus are: (1) a Van Slyke pipette of 100 cc. capacity. The abrupt flare of the sides of the bulb just above the lower stop-cock eliminates pocketing of the equilibrated solution. During the shaking the solution is spread evenly over the entire interior surface of the bulb in a thin layer; (2) a short narrow upper stem equipped with the usual type of stop-cock, cup, and side outlet; (3) a water manometer connected to the upper side outlet; (4) a gas manifold and mercury manometer permitting the introduction of a given partial pressure of  $CO_2$  into the apparatus; (5) a lower outlet through which the apparatus may be washed by suction or samples removed for analysis; (6) a water bath (a 5 gallon jar) which contains the equilibrating pipette only; (7) a metal support for the equilibrator (not shown) adapted to a mechanical shaker, giving a rapid to and fro motion to the free lower end. The axis of rotation is at the level of the upper side arm-water manometer union.

The steps in the determination are as follows: (1) A slight

vacuum is produced in the equilibrator and 5 cc. of the amino acid solution to be equilibrated are drawn in. (2) The solution is equilibrated with air for a minute. (3) The pipette is evacuated and the desired partial pressure of  $\text{CO}_2$  as measured by the mercury manometer is introduced. (4) The pressure in the pipette is brought to that of the atmosphere by letting in air. About half a minute is allowed for the  $\text{CO}_2$  and air to come to the temperature

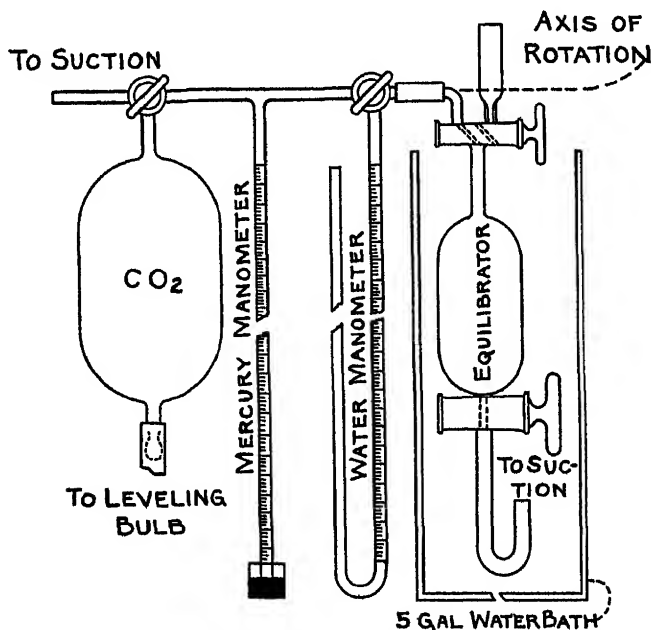


FIG. 3 Apparatus for the measurement of the non-carbonate equilibrium for amino acids and  $\text{CO}_2$ .

of the equilibrator. Inappreciable amounts of  $\text{CO}_2$  are taken up by the solution during this time. (5) The water manometer is connected to the side arm of the equilibrator, and the water menisci in both arms are adjusted to the same level. (6) The equilibration is begun, exactly 45 seconds from the admission of  $\text{CO}_2$ , by rapid motor shaking of the pipette. The changes of the water manometer are recorded, together with the time to the nearest second.

Fig. 4 gives the result of a typical determination on a solution of glycine, 0.1 M, containing 0.05 mole of base. It shows as ordinate the changes in the water level of the manometer and as abscissæ the time as seconds. The curve shows two phases: (a) the first steep phase due to the rapid uptake of  $\text{CO}_2$  in part in physical solution and in part as carbamate; (b) a second slow phase which is due to the slow hydration of  $\text{CO}_2$  to form carbonic acid and its ions  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$ . It is found by experiment that physical solution is complete in about 15 seconds. By extrapolation of the slow phase to this time, we get a point A. At this point the solu-

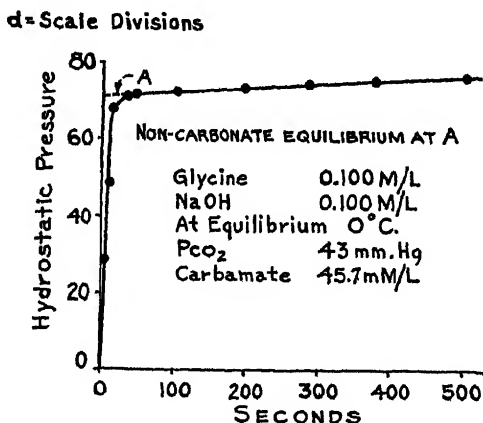


FIG 4 Typical biphasic curve of  $\text{CO}_2$  uptake for the measurement of the non-carbonate equilibrium.

tion is in equilibrium with respect to carbamate and  $\text{CO}_2$  in the virtual absence of  $\text{H}_2\text{CO}_3$ ,  $\text{HCO}_3^-$ , or  $\text{CO}_3^{2-}$ . In other words, A represents a definite equilibrium state in the non-carbonate equilibrium of the amino acid- $\text{CO}_2$  system.

The partial pressure of  $\text{CO}_2$  and the carbamate concentration at point A are calculated as follows:

- $\alpha$  = volume (cc) of water manometer per scale division
- $b$  = factor converting scale divisions to mm. of Hg
- $d$  = difference in hydrostatic levels in scale divisions on the Hg manometer
- $V$  = volume of free space of equilibrator including manometer in cc.



$S$  = volume of solution in cc.

$P_i$  = initial pressure of  $\text{CO}_2$  in mm. of Hg

$P_{\text{CO}_2}$  = pressure of  $\text{CO}_2$  at any given time

$B' = B - W$  = barometric pressure less the aqueous tension in mm. of Hg

$$f = \frac{1}{22.4} \frac{1}{760} \frac{273}{T} = \frac{0.0161}{T}; \text{ converts } PV \text{ to mm}$$

Then it can be shown that at any time

$$P_{\text{CO}_2} = \left(1 + \frac{ad}{B}\right) P_i - \left(\frac{aB'}{V} + b\right) d \text{ mm. of Hg}$$

$$\text{Total CO}_2 \text{ in solution} = (\text{Am}^-) + \alpha_{\text{CO}_2} P_{\text{CO}_2} = f \frac{V}{S} d \left(1 - \frac{ad}{B}\right) \left(\frac{aB'}{V} + b\right)$$

$$(\text{Am}^-) = \text{total CO}_2 \text{ in solution} - \alpha_{\text{CO}_2} P_{\text{CO}_2}, \text{ expressed in mm per liter}$$

Repetition of the experiment with a new value of  $P_i$ , gives a new equilibrium point  $A$ . In this way it is possible to determine a series of values of  $P_{\text{CO}_2}$  and  $(\text{Am}^-)$ , each representing an equilibrium state of carbamate and  $\text{CO}_2$  in the absence of carbonates.

A typical calculation is given from Fig. 4. Extrapolating back along the hydration phase to  $t = 15$  seconds, we arrive at point  $A$ . Here the difference in hydrostatic levels of the water manometer is 72 scale divisions. At this point  $\text{CO}_2$  in the solution phase is present only as free  $\text{CO}_2$  and carbamate, and it represents an equilibrium state involving carbamate and amino acid in the virtual absence of carbonates.

The constants for the apparatus are

$$a = 0.052 \text{ cc. per scale division}$$

$$V = 79.2 \text{ cc}$$

$$b = 0.37 \text{ mm. of Hg per scale division}$$

and for the experiment

$$d = 72; \alpha_{\text{CO}_2} = 0.1$$

$$S = 5 \text{ cc.}$$

$$P_i = 100 \text{ mm. of Hg}$$

$$B = 755 \text{ " " "}$$

$$f = 5 \times 90 \times 10^{-3}$$

$$(aB'/V) + b = 0.86$$

$$f(V/S) ((aB'/V) + b) = 0.726$$

$$\text{Then } P_{\text{CO}_2} = (1 + 0.00065 \times 72)100 - 0.86 \times 72 = 42.7 \text{ mm. of Hg}$$

Total $\text{CO}_2 = (1 - 0.00065 \times 72) 0.726 = 50.0$ mm per liter	
$\alpha_{\text{CO}_2} P_{\text{CO}_2}$	4.3
$(\text{Am}^-)$	45.7 mm per liter
$P_{\text{CO}_2}$	42.7 mm. of Hg

### Method of Calculating $K_{\text{Am}}$

The non-carbonate equilibrium measured in the fashion just described gives, for example, a series of equilibria in the case of

TABLE IV

*Non-Carbonate Equilibrium of Glycine and Alanine with  $\text{CO}_2$*

$t = 20^\circ$ ; alanine 0.1 M; NaOH 0.05 M;  $\text{pK}_z$  9.72;  $\alpha_{\text{CO}_2}$  0.0516 mm per liter per mm. of Hg

Glycine 0.1 M; NaOH 0.05 M;  $\text{pK}_z$  9.75

	Experiment No	$P_{\text{CO}_2}$	$(\text{Am}^-)$	$\frac{(\text{Am}^-)[(N) - (B) + (\text{Am}^-)]}{[(B) - 2(\text{Am}^-)]^2}$
		mm. Hg	mm per l	
Alanine, $K_{\text{Am}} = (2.53 \pm 0.06) \times 10^{-6}$	1	1.3	6.7	0.28
	2	3.2	9.1	0.53
	3	3.1	12.5	1.25
	4	5.4	15.6	2.85
	5	8.2	16.9	4.31
	6	11.3	18.1	6.49
	7	15.0	19.1	9.42
Glycine, $K_{\text{Am}} = (2.67 \pm 0.07) \times 10^{-6}$	1	2.6	6.7	0.21
	2	2.8	9.4	0.57
	3	4.6	12.0	1.10
	4	4.6	12.0	1.10
	5	5.8	14.9	2.36
	6	8.0	17.1	4.59
	7	15.0	19.1	9.43
	8	23.7	20.0	16.00

glycine and alanine as shown in Table IV. Figs. 5, *a* and 5, *b* show the data plotted in two ways:

(a) The left side of Equation 11, *viz.*

$$\frac{(\text{Am}^-)[(N) - (B) + (\text{Am}^-)]}{[(B) - 2(\text{Am}^-)]^2}$$

is plotted against  $P_{\text{CO}_2}$ . A linear relation is found as expected from the equation. From the slope of this line we calculate  $K_{\text{Am}}$  for alanine.

Slope =  $\alpha_{\text{CO}_2} (K_{\text{Am}}/K_g) = 0.68 \pm 0.017$  (s.e.);<sup>4</sup> from which

$$K_{\text{Am}} = (2.53 \pm 0.06) 10^{-6}$$

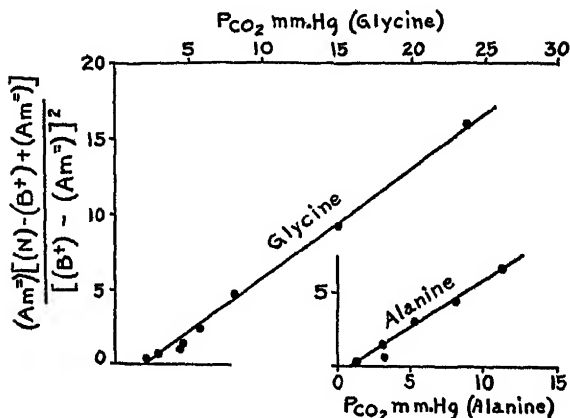


FIG. 5, a

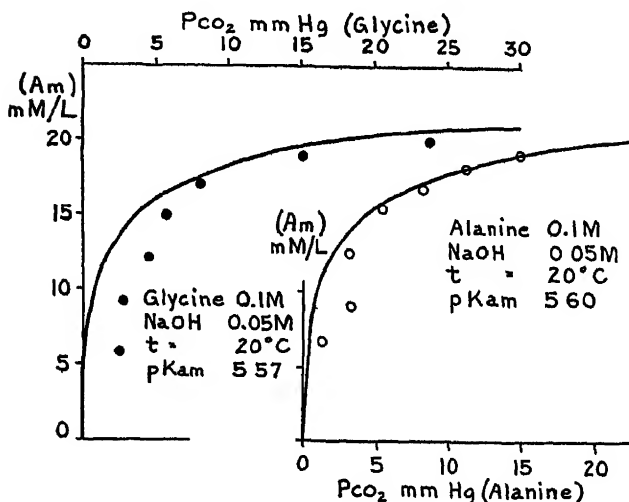


FIG. 5, b

FIGS 5, a AND 5, b The non-carbamate equilibrium of glycine and alanine. Fig. 5, a illustrates the method of calculating  $K_{\text{Am}}$ ; Fig 5, b gives a comparison of calculated and observed values of  $(\text{Am}^-)$ .

<sup>4</sup> s.e. refers to standard error of the mean.

(b) Using this value of  $K_{Am}$  we calculate the full line of Fig. 5, *b* by Equation 11. The figure also gives the experimental points, which show a good fit to the calculated line.

It will be observed that the straight line of Fig. 5, *a* does not pass through the origin as it should. This is undoubtedly due to a constant fixed error in the determination of  $P_{CO_2}$  or  $(Am^-)$ . The relative values of  $Am$  and  $P_{CO_2}$  follow the equation with considerable precision, as indicated by the small standard error of  $K_{Am}$  calculated statistically. In consequence of this displacement of the curve of Fig. 5, *a* to the right, the fit of the calculated curve of Fig. 5, *b* appears less exact.

#### DISCUSSION

We have measured the non-carbonate equilibrium of several amino acids and calculated their carbamate mass action constants under a variety of conditions. All of these equilibria show the same characteristics.

1. A hyperbolic curve of  $(Am^-)$  plotted against  $P_{CO_2}$  rises to a maximum at comparatively low pressures of  $CO_2$ .
2. The maximum  $(Am^-)$  is always one-half of the base.
3.  $\frac{(Am^-) [(N) - (B^+) + (Am^-)]}{[(B) - 2(Am^-)]^2}$  is linear to  $P_{CO_2}$ .

A few such curves are shown for illustration. Figs. 5, *a* and 5, *b* also show the non-carbonate equilibria of 0.1 M glycine with 0.05 M base at 20°. The curve is very similar to that of alanine,  $K_{Am} = (2.67 \pm 0.06) 10^{-6}$ .

Fig. 6 shows the equilibrium of cysteic acid.  $K_{Am}$  is considerably less and in consequence the curve is more stretched out. Fig. 7 shows a series of curves for glycine with varying base. The mean value of  $pK_{Am}$  for all the curves is 5.27. Using this value we have calculated the full curves. It is apparent that the calculated lines conform to the experimental values with considerable precision.

In Table V we have given the values of  $K_{Am}$  determined as outlined above for the three amino acids, glycine, alanine, and cysteic acid, under varying conditions. Several points of interest are worth emphasizing. (1) Glycine and alanine show maximum carbamate at low pressures. The absolute error in  $P_{CO_2}$  may then be considerable, while the relative error which governs the

error of  $K_{Am}$  may be much less. (2) Cysteic acid shows a maximum ( $Am^-$ ) at a high  $P_{CO_2}$ . (3) When the base is high, the

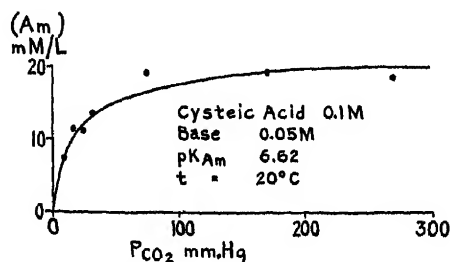


Fig. 6 The non-carbonate equilibrium of cysteic acid, showing the calculated and observed values of ( $Am^-$ ).

Glycine 0.1M  $pK_{Am} = 5.27$   $t = 23^\circ C$ .

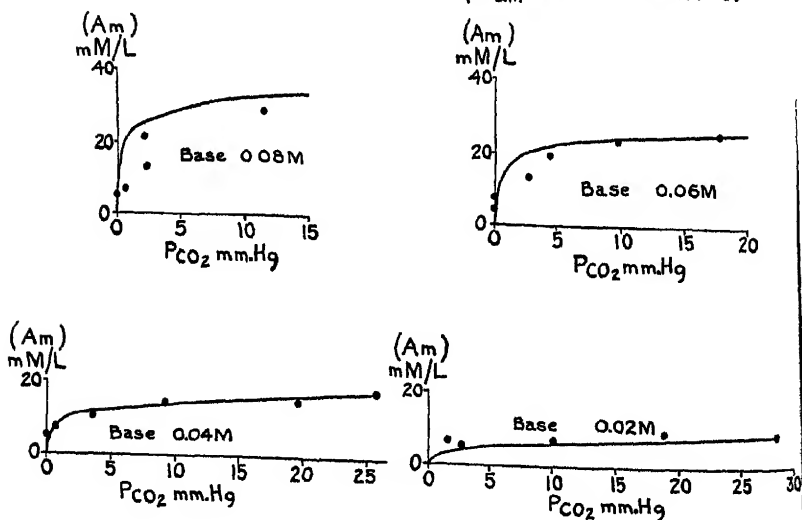


Fig. 7. Non-carbonate equilibrium of glycine, showing the calculated and observed values of ( $Am^-$ ), base varying.

calculation of  $K_{Am}$  is less trustworthy, because the curve is very steep and the measurement of low  $P_{CO_2}$  is subjected to huge errors. (4) When the base is very low, ( $Am^-$ ) is small and its measurement

involves considerable error. (5) 0.5 equivalent of base is about optimum for accurate determinations of  $K_{Am}$ . (6) At high temperatures the hydration of  $CO_2$  is so rapid that the biphasic character of the  $CO_2$  uptake curve (Fig. 4) becomes obscured, and the location of the equilibrium point *A* becomes difficult. (7) The effect of an increase of temperature is to increase  $K_{Am}$ , as is apparent from Table V.

TABLE V  
*Carbamate Mass Action Constant  $pK_{Am}$  for Various Amino Acids Determined by the Non-Carbonate Equilibrium*

<i>t</i>	Amino acid (0.1 M per liter)	Base	$pK_{Am}$
°C		M per l	
20	Cysteic	0.050	6.62
20	"	0.080	6.83
20	"	0.050	6.77
20	"	0.033	6.33
20	Glycine	0.050	5.54
20	Alanine	0.050	5.57
0	Glycine	0.050	5.8
38	"	0.050	4.9
23	"	0.020-0.080	5.47

#### *Total Equilibrium of Amino Acid and $CO_2$*

When the total equilibrium, as we have defined it previously, is to be studied, the solution of amino acid containing known concentrations of the acid and the available base (usually NaOH) is equilibrated for a long period of time (2 to 10 hours). The method employed is the usual tonometric one (Austin *et al.*, 1922). At the end of the equilibration, the solution and the gas phase are separated and analyzed for total  $CO_2$ , carbonates, carbamates,  $P_{CO_2}$ , and  $pH^+$ .

The theoretical development of this equilibrium is based on the same primary assumptions which have already been discussed, and the case is no more complicated in principle than the non-carbonate equilibrium. Since, however, hydration of  $CO_2$  to carbonates is complete, the latter must be included in the mass action equations, which thus become more complex algebraically.

These mass action equations are derived as follows: At equilibrium the following relations hold. The total amino acid is

$$(N) = (Z^-) + (Z^\pm) + (Am^-) \quad (8)$$

The amphanion concentration is (Equations 4 and 8)

$$(Z^-) = \frac{K_z}{K_z + (H^+)} [(Z^-) + (Z^\pm)] = \frac{K_z}{K_z + (H^+)} [(N) - (Am^-)] \quad (12)$$

The base as carbonate is

$$(B_{On}) = \frac{\alpha_{CO_2} K_1 P_{CO_2}}{(H^+)} \left( 1 + \frac{2K_2}{(H^+)} \right) \quad (1)$$

Then neglecting  $(OH^-)$ , which at  $pH^+ < 12$  is small, we get for the total available base (Equations 1, 6, 12)

$$\begin{aligned} (B^+) &= (Z^-) + 2(Am^-) + (B_1HCO_3^-) + 2(B_2CO_3^{2-}) \\ &= \frac{K_z}{K_z + (H^+)} [(N) - (Am^-)] + 2(Am^-) + \frac{\alpha_{CO_2} K_1 P_{CO_2}}{(H^+)} \left( 1 + \frac{2K_2}{(H^+)} \right) \end{aligned} \quad (13)$$

For the total amino acid we have (Equation 9) as before

$$(Am^-) = \frac{(N)}{\left[ 1 + \frac{(H^+)}{\alpha_{CO_2} P_{CO_2} K_{Am}} \left( 1 + \frac{(H^+)}{K_z} \right) \right]} \quad (9)$$

As before, Equation 9 is an expression for the first assumption as to the mode of combination of  $CO_2$ . It may be tested for its validity in the case of the total equilibrium if the  $(Am^-)$ ,  $P_{CO_2}$ , and  $(H^+)$  are known for a series of equilibria. In that case (if  $K_z$  has been determined independently) it will be found that  $K_{Am}$  is a constant, as we shall show. Furthermore, its value will be found to be of the same order as that found in the non-carbonate equilibrium.

Equation 13 will be recognized to contain the second of our assumptions. It too will be found to be in satisfactory agreement with experiment. It may be tested for its validity provided a full knowledge of the values of the variables  $(Am)$ ,  $P_{CO_2}$ , and  $(H^+)$  are known for a series of total equilibria. It will be noted that the equation is independent of  $K_{Am}$ . The constants  $K_z$ ,

$K_1$ , and  $K_2$  are, of course, determinable by methods totally independent of hypotheses of carbamate combination.

The two equations may be combined with the elimination of one of the variables ( $P_{\text{CO}_2}$ ). The resultant quadratic may be simplified to a close approximation equation. No new hypothesis is introduced by this step. The equation when  $\alpha_{\text{CO}_2} P_{\text{CO}_2}$  is eliminated turns out to be

$$(\text{Am}^-) = c/b \quad (14)$$

where

$$b = (\text{B}^+) + 2(N) \left[ \left( 1 - \frac{K_z}{K_z + (\text{H}^+)} \right) \right] + \frac{1 + \frac{2K_2}{(\text{H}^+)}}{\frac{K_z}{K_z + (\text{H}^+)}} \frac{K_1}{K_{\text{Am}}}$$

$$c = (N)^2 \left[ \frac{(\text{B}^+)}{(N)} - \frac{K_z}{K_z + (\text{H}^+)} \right]$$

These equations, which will be amply shown to agree with experiment, complete the theory and it is now possible to calculate in the case of the total equilibrium the concentration of carbamate as a function of  $\text{pH}^+$ ,  $P_{\text{CO}_2}$ , or total bound  $\text{CO}_2$  for any amino acid solution when the constants  $K_1$ ,  $K_2$ ,  $K_z$ , and  $K_{\text{Am}}$  are known together with the composition as given by  $(N)$  and  $(\text{B}^+)$ .

The steps in such a calculation are: (1) Calculate  $(\text{Am}^-)$  at a given  $\text{pH}^+$  by Equation 14. (2) Calculate  $(Z^-)$  by Equation 12. (3) Calculate  $(\text{B}_{\text{On}})$  by Equation 13. (4) Calculate  $(\text{On})$ , the total carbonate, by the combined Equations 1 and 2

$$(\text{On}) = (\text{B}_{\text{On}}) \frac{1 + \frac{2K_2}{(\text{H}^+)}}{1 + \frac{K_2}{(\text{H}^+)}} \quad (15)$$

(5) Calculate  $P_{\text{CO}_2}$  by Equation 2

$$P_{\text{CO}_2} = \frac{(\text{On}) \frac{(\text{H}^+)}{K_1}}{\alpha_{\text{CO}_2} \left[ 1 + \frac{K_2}{(\text{H}^+)} \right]}$$



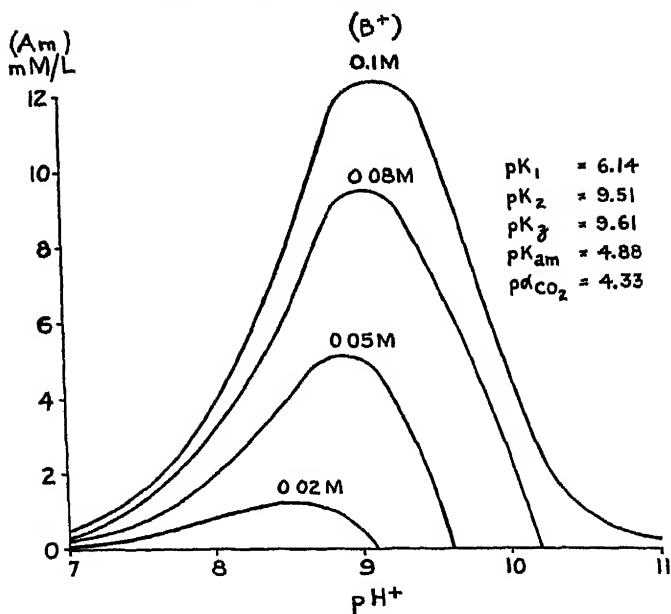


FIG. 8, a

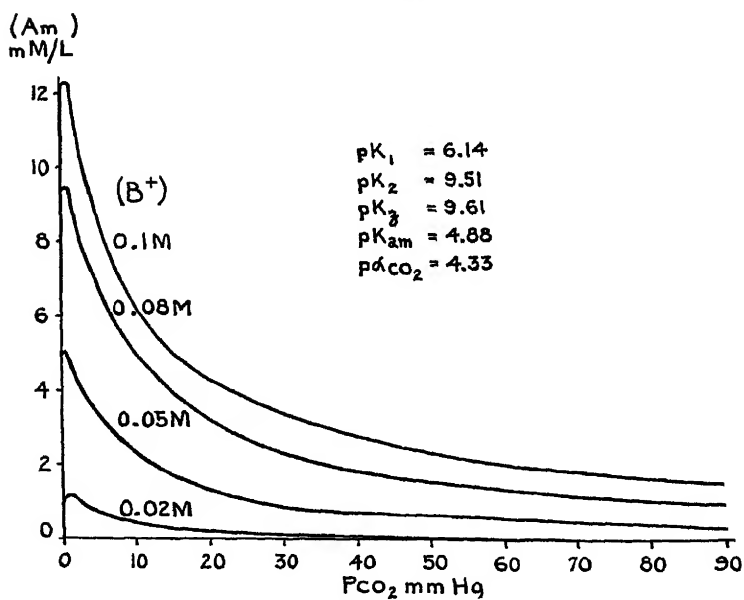


FIG. 8, b

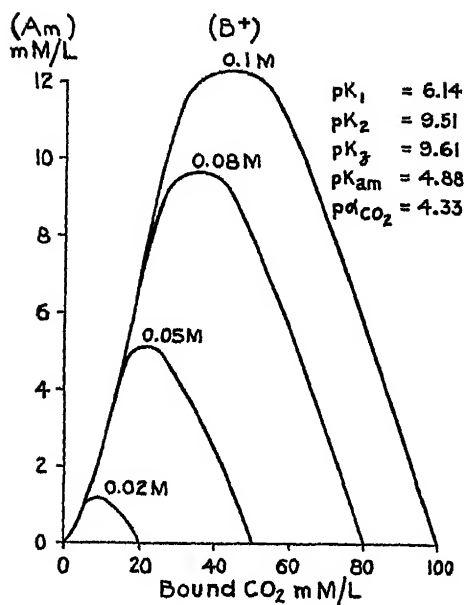


FIG. 8, c

FIGS. 8, a, 8, b, AND 8, c Calculated total equilibrium of glycine and  $CO_2$  with varying available base. Fig. 8, a, carbamate concentration as a function of  $pH^+$ ; Fig. 8, b, carbamate concentration as a function of  $P_{CO_2}$ ; Fig. 8, c, carbamate concentration as a function of total bound  $CO_2$ .

We have made such a calculation in the case of 0.1 M glycine where the base varied from 0.2 to 1.0 M, employing the constants indicated. The results are shown in Figs. 8, a, 8, b, and 8, c where we have plotted the carbamate concentration as a function of  $pH^+$ ,  $P_{CO_2}$ , and total bound  $CO_2$ . The following characteristics may be emphasized (1) The initial  $pH^+$  is dependent on the available base present and is given by the equation

$$pH^+ = pK_z + \log \frac{(B^+)}{(N) - (B^+)}$$

(2) As the  $pH^+$  decreases owing to the increase of  $P_{CO_2}$ , there is a rise to a maximum value of carbamate which is greater the greater the base concentration. (3) The maximum carbamate is about

20 per cent of the available base, rather than 50 per cent, as in the case of the non-carbonate equilibrium. (4) The maximum carbamate is found at very low pressures of  $\text{CO}_2$ . (5) As the  $P_{\text{CO}_2}$  is further increased, the carbamate decreases and becomes zero at the isoelectric point of the amino acid.

We believe that the above application of the mass action law is the first complete analysis of the total amino acid- $\text{CO}_2$  equilibrium. We shall subsequently show that it is in complete accord with experiment.

We have previously (Stadie, 1935) pointed out the necessity of differentiating the non-carbonate from the total equilibrium of amino acids and  $\text{CO}_2$ . Failure to do so may lead to misinterpretation of experiments both on amino acids and hemoglobin.

We have not discussed the effect of interionic action upon the equilibrium nor have we any experiments bearing upon it. The mass action constants included in all our equations are valid only for the particular thermodynamic environment given and perhaps vary considerably from one solution to another.

It might be thought that our equations and the curves of Fig. 8, *a* are the same as those of Faurholt (1925). This is not the case, for Faurholt's analysis was incomplete and might readily be misleading. The close student of his paper will note the following (*cf* p. 28). (1) Faurholt's primary assumption is the same as our first; *i.e.*,  $\text{CO}_2$  combines as carbamate only with  $\text{COO}^- \cdot \text{R} \cdot \text{NH}_2$  and not with  $\text{COO}^- \cdot \text{R} \cdot \text{NH}_3^+$ . (2) Carbamate is regarded as binding 1 equivalent of base rather than 2. (3) No statement is made as to the partition of the base at equilibrium equivalent to our Equation 13. (4) In consequence, his equilibrium equation lacks the important constants ( $N$ ) and ( $B^+$ ). (5) The equation cannot be solved for a particular case. His curves (Figs 1 to 4) are for cases where the amino acid not combined with  $\text{CO}_2$  as carbamate is constant and the *total amino acid* is varying. (6) Further, the  $P_{\text{CO}_2}$  cannot be calculated, since ( $B^+$ ) is omitted. It has an indeterminate value which is only made definitive by assigning a value to ( $B^+$ ).

### *Experimental Proof*

The proof of the above equations for expressing the total equilibrium of amino acids and  $\text{CO}_2$  is contained in a series of experi-

ments on glycine containing varying quantities of available base equilibrated as described above for the determination of the total equilibrium.

In the first experiment 0.1 M glycine together with 0.1 M NaOH and 0.1 M NaCl was used. The equilibrated solutions were analyzed for total  $\text{CO}_2$ ,  $P_{\text{CO}_2}$ ,  $\text{pH}^+$ , and carbamate. These results are given in Table VI.

TABLE VI

*Total Carbamate Equilibrium of Glycine and  $\text{CO}_2$* 

$t = 25^\circ$ ; initial solution, glycine, NaOH, NaCl, each 0.100 M per liter;  $\alpha_{\text{CO}_2} = 0.0472$  mm per liter per mm of  $\text{Hg}_2$ .

Experiment No	$P_{\text{CO}_2}$ observed	$\text{pH}^+$ observed	Total $\text{CO}_2$ observed	$\alpha_{\text{CO}_2} P_{\text{CO}_2}$ calculated	Bound $\text{CO}_2$ calculated	(Am <sup>-</sup> ) observed	(On) calculated
	mm Hg		mm per l.	mm per l	mm per l	mm per l	mm per l
1	95.4	7.60	101.3	4.51	96.8	1.5	95.3
2	58.7	7.72	98.5	2.77	95.7	1.6	94.1
3	30.5	7.93	94.9	1.44	93.5	3.0	90.5
4	4.0	8.63	75.1	0.19	74.9	8.3	66.6
5	1.8	8.98	63.5	0.09	63.4	13.5	49.9
6	<0.1	8.99	60.2	0.00	60.2	13.0	47.2
7	<0.1	8.99	60.2	0.00	60.1	14.6	45.5
8	0.6	9.25	49.1	0.03	39.1	8.8	40.2
9	<0.1	9.65	32.0	0.00	32.0	8.3	23.7
10	<0.1	9.90	24.3	0.00	24.3	7.5	16.8
11	<0.1	9.93	23.0	0.00	23.0	7.2	15.8
12	<0.1	10.25	14.1	0.00	14.1	4.1	10.0

*Proof of the Second Assumption, Equation 13*—It is possible to calculate the equivalents of base as glycinate carbamates and carbonate in the solution at equilibrium in each case by employing *direct* experimental values as follows:

$$(\text{Z}^-) = \frac{K_z}{K_z + (\text{H}^+)} [(\text{N}) - (\text{Am}^-)] \quad (12)$$

$$(\text{B}_{\text{O}_n}) = (\text{On}) \frac{1 + \frac{K_2}{(\text{H}^+)}}{1 + \frac{2K_2}{(\text{H}^+)}} \quad (15)$$

where  $(On) = \text{total bound } CO_2 - (Am^-)$ . The base bound as carbamate is then given as  $(B_2Am^-) = (B^+) - [Z^- + (B_{On})]$ . The ratio of this to  $(Am^-)$  is given in each case in Table VII. The mean value of this ratio is  $1.8 \pm 0.3$  (s.e.). In accordance with the hypothesis that the carbamates are dibasic, this value does not significantly differ from 2.

*Proof of the First Assumption*—The second column of Table VIII shows the value of  $pK_{Am}$  calculated by Equation 9, which

TABLE VII

*Ratio of Base Bound by Carbamate to Total Carbamate in Total Equilibrium with  $CO_2$*

Experiment No	Ratio
1	1.7
2	2.0
3	1.7
4	2.3
5	1.9
6	1.9
7	1.9
8	2.0
9	1.8
10	1.4
11	1.4
12	(0.9)
Mean . . . . .	$1.8 \pm 0.3$ (s.e.)

contains the first assumption only.  $pK_{Am}$  is constant over a wide range of  $P_{CO_2}$  and  $pH^+$ .

Equation 14, in which both assumptions are involved, offers a better method of calculating  $pK_{Am}$ . The mean value (last column of Table VIII) is 4.81, and the constancy over a wide range of  $pH^+$  is regarded as proof that the assumptions made are correct.

We may now extend the theory to other cases where the base is varied. Solutions of 0.1 M glycine containing 0.1 M, 0.08 M, and 0.05 M respectively of NaOH were equilibrated with  $CO_2$  and analyzed for total  $CO_2$ ,  $P_{CO_2}$ , and carbamates. The resulting data are plotted in Figs. 9 to 11. In each figure the full lines are

calculated with the constants indicated. In all cases experiment is in substantial agreement with the expected values.

We regard this agreement as proof that the theory of the amino acid-carbamate- $\text{CO}_2$  equilibrium outline here is essentially correct.

*Note on the Ferguson-Roughton (1934) Carbamate Method*

The determination of the carbamates in the presence of carbonate was carried out successfully by this method. In brief, it depends upon the precipitation of free  $\text{CO}_2$  and carbonates as

TABLE VIII

*Calculation of Carbamate Mass Action Constant for Total Equilibrium of Glycine with  $\text{CO}_2$*

Experiment No.	$\text{pK}_{\text{Am}}$ (Equation 9)	$\text{pK}_{\text{Am}}$ (Equation 14)
1	5.07	4.94
2	5.07	5.01
3	4.91	4.91
4	4.92	4.93
5	4.85	4.78
6		4.65
7		4.69
8	5.18	5.02
9		4.98
10		4.65
11		4.69
12		4.51
Mean.....	5.00	4.81
S.E.....	0.55	0.52

$\text{BaCO}_3$  at optimum pH (ca. 11 to 11.5), leaving the soluble carbamates in solution. The centrifuged supernatant liquid from the precipitated carbonate is then analyzed in 5 cc. samples for  $\text{CO}_2$ , which represents the carbamates of the original solution.

We have found that the precautions advocated by Ferguson and Roughton are quite necessary, particularly the rapid mixing of reagents by means of a Hartridge and Roughton (1923) mixing chamber, the approximate adjustment of the terminal  $\text{pH}^+$ , and the need of constant total  $\text{CO}_2$  of the reaction mixture.

Three 5 cc. Luer syringes are connected to a 0.05 cc. Hartridge chamber by means of three radially placed 0.1 mm. jets. By a suitable device the three plungers can be pushed in simultaneously and the contents of the syringes mixed within a small fraction of a second. The three syringes contain (a) the solution of amino acid,

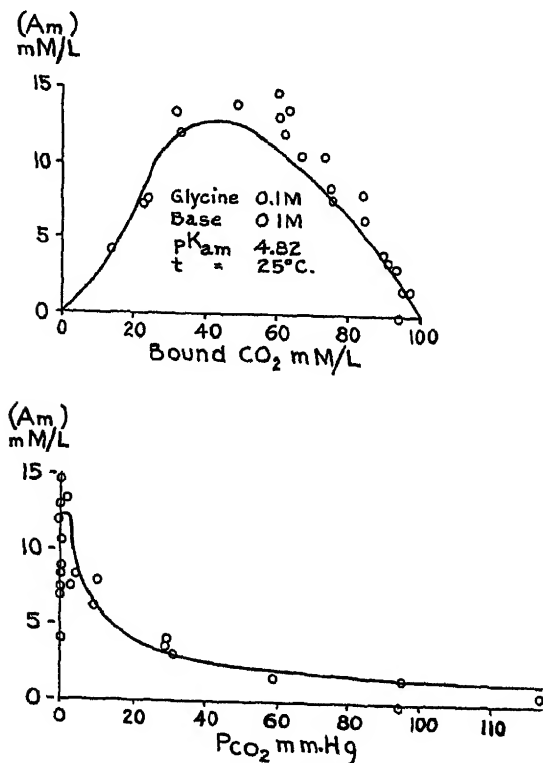


FIG 9. Total equilibrium of carbamate and glycine (base = 0.1 M), showing calculated and observed values.  $pK_1$ ,  $pK_2$ ,  $pK_z$  used, the same as in Fig 8, a

carbonates, and carbamates; (b)  $\text{Na}_2\text{CO}_3$  sufficient to bring the total  $\text{CO}_2$  in (a) up to 50 mm per liter; (c) 0.5 M  $\text{BaCl}_2$  and sufficient  $\text{NaOH}$  to make the total available base in (a) equal to twice the total  $\text{CO}_2$  + the amino acid + 50 mm per liter extra. This gives a final reaction mixture which in all cases is approximately the

same and is optimum for the complete precipitation of the carbonates.

In the absence of amino acids there is no difficulty in precipitating all the free  $\text{CO}_2$  in a solution as  $\text{BaCO}_3$ . In the presence of amino acid the situation is altogether different, and we have repeatedly found that a considerable part of the free  $\text{CO}_2$  is not

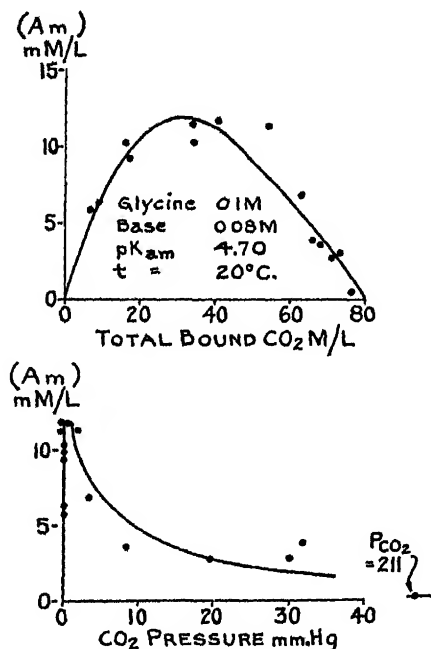


FIG 10. Total equilibrium of carbamate and glycine (base = 0.08 M), showing calculated and observed values  $pK_1$ ,  $pK_2$ ,  $pK_z$  used, the same as in Fig. 8, a

precipitated. A representative experiment to show this appears in Table IX. The reason for the failure of 60 per cent of the free  $\text{CO}_2$  to precipitate as  $\text{BaCO}_3$  is clear. It must first be hydrated to carbonate, but this reaction is very slow. When the reaction mixture is suddenly made alkaline, a considerable fraction of the free  $\text{CO}_2$  rapidly combines with the amino acid present to form carbamates and escapes precipitation.



If the free  $\text{CO}_2$  is in the original solution in (a) together with amino acid at low pH (5 to 7), the same result is obtained and for the same reason. The amount of free  $\text{CO}_2$  not precipitated is roughly proportional to the total. It might erroneously be thought to be carbamate and to represent a mode of combination

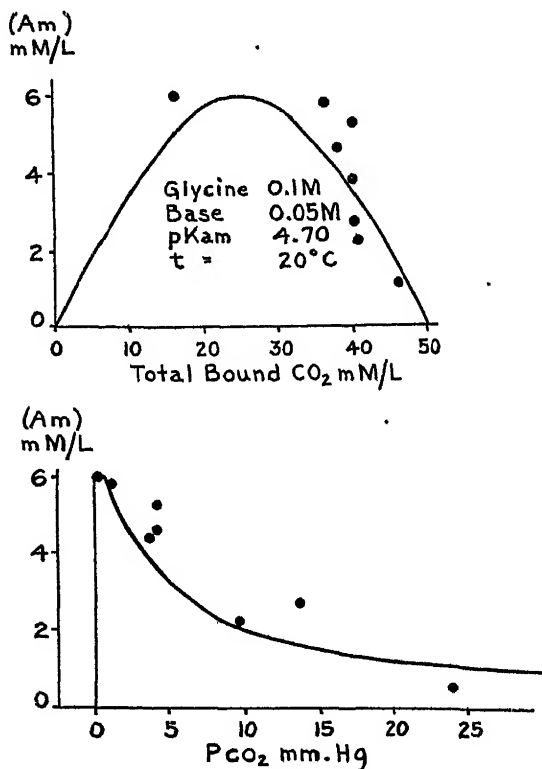


FIG. 11 Total equilibrium of carbamate and glycine (base = 0.05 M), showing calculated and observed values  $pK_1$ ,  $pK_2$ ,  $pK_3$  used, the same as in Fig 8, a

of  $\text{CO}_2$  and amino acid in the acid range different from that in the alkaline range. Thus result we have excluded by the experiments shown in Tables I and II.

Thus difficulty in the complete precipitation of the free  $\text{CO}_2$  by the Ferguson-Roughton method we have completely overcome

by adding to syringe (b) sufficient carbonic anhydrase to accelerate the hydration of  $\text{CO}_2$  200- or 300-fold.

Then the free  $\text{CO}_2$  in syringe (a), when suddenly made alkaline by admixture with the contents of (b) and (c) in the Hartridge mixer, is very rapidly converted into carbonate and precipitated as  $\text{BaCO}_3$ . We used potent preparations of carbonic anhydrase prepared as previously described (Stadie and O'Brien, 1933).

Table IX shows how effectively large amounts of free  $\text{CO}_2$  are hydrated to carbonate and precipitated in the presence of sufficient carbonic anhydrase.

The experiments of Tables I and II also show the rapid hydration and precipitation of large amounts of free  $\text{CO}_2$  by this method.

TABLE IX

*Precipitation of Free  $\text{CO}_2$  by Ferguson-Roughton Method with and without Carbonic Anhydrase*

Syringe (a) contains 30 mm per liter of free  $\text{CO}_2$ ; syringe (c) 0.5 M  $\text{BaCl}_2$  and 0.11 M  $\text{NaOH}$ .

Syringe (b)	Acceleration of hydration of $\text{CO}_2$ by anhydrase	$\text{CO}_2$ not precipitated
		<i>mm per l</i>
Water	None	0.0
0.1 M glycine	"	18.0
0.1 " "	100-fold	0.4
0.1 " "	100-fold	0.5
0.1 " "	50-fold	0.1
0.1 " "	25-fold	8.2

## SUMMARY

1. The characteristics of the combination of  $\text{CO}_2$  with amino acids to form carbamates are given; the data in the literature are supplemented by new experiments.

2. Evidence is given to show that it is the amphanion  $\text{COO}^- \cdot \text{R} \cdot \text{NH}_2$  and not the zwitter ion  $\text{COO}^- \cdot \text{R} \cdot \text{NH}_3^+$  of amino acids that combines with  $\text{CO}_2$  to form carbamates.

3. The ionization of carbamates is discussed and proof is given that they are dibasic salts at  $\text{pH} > \text{about } 7$ .

4. The assumptions are stated which permit the development of mass action equations for the carbamate equilibrium of amino acids and  $\text{CO}_2$  in aqueous solutions.

5. It is shown that two types of equilibria must be considered; *viz*, (a) the "non-carbonate" equilibrium, when no carbonates are present; (b) the "total" equilibrium, when carbonates as well as carbamates are present.

6. Mass action equations are developed which represent both equilibria. Typical curves are drawn in both cases and the characteristics of the equilibria are outlined.

7. Ample experimental evidence is given to support the equations. Mass action constants for the amino acid-carbamate equilibrium are calculated under a variety of conditions.

8 By the use of carbonic anhydrase the Ferguson-Roughton carbamate method can be used when large amounts of free  $\text{CO}_2$  are present. We exclude by this method any mode of combination of  $\text{CO}_2$  and amino acid peculiar to the acid range ( $\text{pH}^+ 5$  to 7).

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# THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

## XLII. STUDIES ON PHTHIOIC ACID\*

By M. A. SPIELMAN† AND R. J. ANDERSON

(From the Department of Chemistry, Yale University, New Haven)

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### INTRODUCTION

It has been shown by work in this laboratory that the human type of tubercle bacillus, Strain H-37, contains a series of new saturated fatty acids, of high molecular weight, which are either liquid at room temperature or are low melting solids (1, 2). Although these acids are saturated compounds, their lead salts are easily soluble in ether.

The first member of this series of new acids was called tuberculostearic acid (2) and the formula  $C_{18}H_{36}O_2$  was assigned to it. The second member was called phthioic acid (2) and its composition corresponded to that of a hexacosanic acid,  $C_{26}H_{52}O_2$ . Phthioic acid is biologically and optically active; the purest specimen previously described had a specific rotation of  $+11.9^\circ$  (3). A third acid was also isolated, which had a levorotation and a higher molecular weight than phthioic acid (3).

It has been shown by the work of Sabin (4) and collaborators that phthioic acid acts as a maturation factor for monocytes and epithelioid cells and that on injection into normal animals it causes the formation of typical tubercular tissue. The highly purified phthioic acid described in the present report was also tested physiologically in Dr. Sabin's laboratory at the Rockefeller Institute and was found to possess the same biological activity as the former less pure preparations. For this and other reasons

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† National Research Council Fellow in Chemistry

the chemical constitution of phthioic acid presents a problem of particular interest.

In order to gain some information concerning the structure of phthioic acid some preliminary experiments have been made and the results, although far from conclusive, are reported at this time. In the first place the acid was carefully purified by fractional distillation of the methyl ester until the specific optical rotation was constant. The highest value attained was  $+12.2^\circ$  and this value could not be changed by further distillation. The analytical data agreed with the formula  $C_{27}H_{54}O_2$  which is that of the methyl ester of a hexacosanic acid.

Saponification of the methyl ester yielded an acid which was liquid in a warm room but which solidified readily on cooling and melted at  $20-21^\circ$ ,  $[\alpha]_D = +12.56^\circ$ . Analysis and determination of molecular weight confirmed the formula  $C_{26}H_{52}O_2$ , which was first advanced. The search for a suitable solid derivative was not particularly successful; the amide proved to be the best.

Tuberculostearic acid, with which phthioic acid occurs, was thought at first to have the formula  $C_{18}H_{36}O_2$  but has been shown by Spielman (5) to be 10-methylstearic acid,  $C_{19}H_{38}O_2$ . By inference one might also expect phthioic acid to be a methyl or polymethylated fatty acid. In speculating about the position of the methyl groups the conclusion is obvious that one of them is near to the carboxyl group. Levene, Rothen, and Marker (6) have examined the optical properties of methyl derivatives of fatty acids and found that the molecular rotations diminished as a methyl group is moved successively farther from the carboxyl group. The relatively high molecular rotation,  $+50^\circ$ , of phthioic acid would indicate a methyl group in the  $\alpha$ ,  $\beta$ , or  $\gamma$  position, preferably in the  $\alpha$  position.

In order to determine whether phthioic acid is substituted in the  $\alpha$  position we tried the chlorination reaction with phosphorus pentachloride on the N-substituted amide as developed by von Braun *et al* (7) but the results were ambiguous, chlorine numbers (8) of 140, 220, and 480 being obtained, whereas the theoretical values for primary and secondary acids are 200 and 100 respectively.

Another method of approach was tried; namely, the degradation reactions as used by Wieland and his associates (9) on cho-

lanic acid. An  $\alpha$ -methyl acid should lead to a ketone, while a  $\beta$  or a  $\gamma$  acid should yield an acid. The reaction product consisted mainly of a neutral substance which gave a faint but positive iodoform reaction and which formed a semicarbazone. However, the substance was undoubtedly a mixture and a pure semicarbazone could not be isolated. The evidence in this case, while inconclusive, tends to favor the presence of an  $\alpha$ -substituting group.

In the hope of locating other branches in the chain, phthioic acid was subjected to drastic oxidation after the fashion used in the investigation of tuberculostearic acid (5). A steam-volatile acid was isolated, which, after purification as the *p*-bromophenacyl ester, corresponded to the formula  $C_{11}H_{22}O_2$ . Neither the acid nor its derivatives were identical with *n*-undecylic acid; hence it must have a branched chain. No dicarboxylic acids could be found in the oxidation mixture.

Further experiments toward the elucidation of the structure will have to be postponed until larger quantities of phthioic acid become available for chemical investigations.

#### EXPERIMENTAL

About 62 gm. of a mixture of higher acids were available for purification. The acid, which had been accumulated during several years, had been isolated from the acetone-soluble fat of the human tubercle bacillus, Strain H-37. The rotation of the fractions varied from  $+2^\circ$  to  $+11.9^\circ$ . These fractions had been purified by repeated fractionation in a high vacuum of the methyl esters; hence they were entirely free from tuberculostearic acid whose methyl ester has a much lower boiling point, the low dextrorotation being due to the presence of the levorotatory acid described in a former publication.

*Attempt to Purify Phthioic Acid by the Twitchell Lead Salt Method*—During the purification of tuberculostearic acid it was observed by Spielman (5) that its lead salt, although very soluble in ether, was only slightly soluble in alcohol. We found that the lead salt of phthioic acid is also slightly soluble in alcohol and that the lead salt of the levorotatory acid was practically insoluble in alcohol. An attempt was therefore made to remove the levorotatory acid from the crude phthioic acid by fractional precip-

itation from alcohol with lead acetate, according to the Twitchell (10) procedure. A long and laborious series of fractionations was carried out but the results were disappointing. Starting with an acid,  $[\alpha]_D = +9^\circ$ , we isolated after numerous partial precipitations a small quantity of an acid having  $[\alpha]_D = +11^\circ$ , while the least soluble lead salt gave an acid having  $[\alpha]_D = +2^\circ$ ; the greater portion of the fractions was intermediate between these values. While considerable separation had been effected, the procedure was too slow and incomplete to be suitable for the purification of phthioic acid.

*Attempt to Purify Methyl Esters of Crude Phthioic Acid by Distillation in a Molecular Still*—A molecular still was constructed<sup>1</sup> and connected, by means of a trap which could be cooled in liquid air, to a vacuum line capable of maintaining a pressure of about 0.0001 mm. A mixture of acids with rotations from  $+2^\circ$  to  $+9^\circ$  was methylated and the ester was distilled slowly into four fractions. The temperature of the outside heating element was about  $120^\circ$  and the inside temperature measured by a thermometer placed in a well filled with mercury was about  $60^\circ$ . The rotations of the fractions were as follows: Fraction I,  $+8.8^\circ$ , Fraction II,  $+7^\circ$ , Fraction III,  $+5.5^\circ$ , Fraction IV,  $-1^\circ$ . Further experiments showed that the molecular still could not be relied upon for any efficient separation of the dextro- and levorotatory esters.

*Separation of Methyl Esters by Distillation in Vacuo*—The final fractionation of the mixed esters was carried out in an especially constructed distilling flask consisting of a 20 cc. Pyrex bulb surmounted by a 6 cm. column of the Widmer (11) type but without the inner spiral. The thermometer was held in a sealed-in well filled with mercury and an open side arm of the flask served for the introduction of the sample, while during the distillations there was inserted into this arm and held in place by a piece of heavy pressure tubing, a capillary tube drawn to a very fine point, the tip reaching to the bottom of the flask. The system was evacuated by means of a diffusion mercury pump backed by an efficient Hyvac pump. The pressure was read on a McLeod gage.

The crude methyl esters were distilled in portions of 10 to 12 gm. Specific rotations were determined on each fraction and thus

<sup>1</sup> We are indebted to Dr. Gosta Åkerlöf for assistance in designing this still.

the purification progress was readily followed but the process was long and tedious. The dextrorotation of the top fractions rose rapidly during the first few fractionations to  $+10^\circ$  or  $+11^\circ$  but after that more slowly. The final and constant value of  $+12.2^\circ$  was reached after some fifteen fractionations. This represents apparently the limit of purification because three further distillations caused no change and three different cuts of the last fractionation all had the same rotation. About 12 gm. of the pure ester were obtained. The intermediate fractions were saved for the isolation of the levorotatory acid.

*Properties of Methylphthioate*—The boiling point of methylphthioate varies somewhat with the method of distillation; it is  $158^\circ$  at 0.003 mm. pressure with the oil bath at  $208^\circ$  and when the rate of distillation is 4 to 6 drops per minute. This rate was found to give the most efficient fractionation. With the bath at  $220^\circ$  and at 0.05 mm. pressure the boiling point was  $175\text{--}178^\circ$ .

The purified ester was very faintly yellowish in color. The molecular still gave a colorless distillate, hence the ester was finally distilled in this manner.

The following constants were determined:  $n_D^{25}$ , 1.4550,  $d_4^{25}$ , 0.8620

*Rotation*—0.6145 gm. of ester dissolved in ether and diluted to 10 cc. gave in a 1 dm. tube an average reading of  $+0.75^\circ$ . Hence,  $[\alpha]_D^{25} = +12.2^\circ$ .

*Saponification*—0.5168 gm. of ester required 12.62 cc. of 0.1 N KOH

$C_{27}H_{34}O_2$  Calculated, mol. wt. 410, found, 409.3

*Analysis*—0.01540 gm. substance · 0.01798 gm.  $H_2O$  and 0.04478 gm.  $CO_2$

$C_{27}H_{34}O_2(410)$  Calculated C 79.02, H 13.17

Found " 79.30, " 13.06

*Phthionic Acid*—The purified ester was saponified by refluxing with alcoholic potassium hydroxide. The solution was diluted with water and extracted with ether in order to remove any unchanged ester but only a trace of material remained on evaporation of the ether. The solution was acidified and the acid was extracted with ether. The ethereal solution was washed with water, dried over sodium sulfate, filtered, and the ether was distilled off. The acid, after it had been dried in a vacuum desiccator, formed a pale yellow, viscous, oil at room temperature but



it solidified on cooling and melted in a capillary tube at 20–21°.  $n_D^{25}$ , 1.4628;  $d_4^{25}$ , 0.8763.

*Rotation*—0.5255 gm. of acid dissolved in ether and diluted to 10 cc gave in a 1 dm. tube an average reading of +0.66°. Hence,  $[\alpha]_D = +12.56^\circ$ .

*Molecular Weight*—The acid was dissolved in neutral alcohol and titrated with alcoholic potassium hydroxide, with phenolphthalein as indicator.

0.2200 gm. and 0.3335 gm. of acid required 5.56 cc. and 8.43 cc. of 0.1 N KOH

$C_{26}H_{52}O_2$ . Calculated, mol. wt. 396; found, 395.7, 395.6  
*Analysis*—0.01481 gm. substance · 0.01715 gm.  $H_2O$  and 0.04265 gm.  $CO_2$   
 $C_{26}H_{52}O_2$  (396)      Calculated    C 78.78, H 13.13  
    Found.            " 78.54, " 12.95

*Phthioamide*—Phthioic acid (1.0 gm.) was converted to the acid chloride with thionyl chloride and poured slowly with stirring into 15 cc. of ice-cold aqueous ammonia. The product, which was formed in nearly quantitative yield, was extremely soluble in all organic solvents. The substance was recrystallized three times from methyl alcohol, giving microscopic platelets, m.p. 45°.

*Analysis*— $C_{26}H_{53}ON$  (395)      Calculated    C 78.98, H 13.41  
    Found            " 79.21, " 13.51

*Methylphthioamide*—This derivative was prepared by adding the acid chloride to a cold ethereal solution of methylamine. The solution was washed with dilute potassium hydroxide, dilute hydrochloric acid, and with water. A pale yellowish oil which soon solidified was obtained on the evaporation of the ether; m.p. 27°.

*Analysis*— $C_{27}H_{55}ON$  (409).      Calculated.    C 79.22, H 13.44  
    Found.            " 78.81, " 13.52

*Oxidation of Phthioic Acid*—2 gm. of phthioic acid were oxidized with 3 gm. of chromic acid in 50 cc. of 95 per cent acetic acid in the manner described for tuberculostearic acid (5). The separation of the products of oxidation into fractions was also performed in the same way except that the steam distillation was continued until 1500 cc. of distillate had been collected.

The steam-volatile acid weighed 0.095 gm. and it had a faint odor somewhat similar to that of butyric acid. The acid did not solidify even when cooled in an ice-salt mixture. The *p*-bromophenacyl ester was prepared according to Judefind and Reid (12). The substance after three recrystallizations melted at 49–50°.

*Analysis*—0.01338 gm. substance: 0.00835 gm. H<sub>2</sub>O and 0.02902 gm CO<sub>2</sub>  
 C<sub>19</sub>H<sub>27</sub>BrO<sub>2</sub>(383.1). Calculated. C 59.51, H 7.10  
 Found. " 59.15, " 6.98

Another sample of the acid was converted into the 2,4,6-tribromoanilide and purified as described by Robertson (13). The substance melted at 111°.

*Analysis*—0.01586 gm. substance: 0.00709 gm. H<sub>2</sub>O and 0.02360 gm CO<sub>2</sub>  
 C<sub>17</sub>H<sub>24</sub>Br<sub>3</sub>ON(498). Calculated C 40.96, H 4.81  
 Found. " 40.58, " 5.00

The analytical values of the derivatives mentioned above agree with the calculated values for a saturated C<sub>11</sub> acid but the melting points are lower than those of derivatives prepared from *n*-undecylic acid. A sample of the *p*-bromophenacyl ester of undecylic acid was synthesized and was found to melt at 69°. The 2,4,6-tribromoanilide of *n*-undecylic acid, according to Robertson (13), melts at 129°. It is evident therefore that the volatile acid obtained by oxidation of phthioic acid is not *n*-undecylic acid.

From the non-volatile material of the oxidation mixture, after the steam distillation, about 0.5 gm. of apparently unchanged phthioic acid was isolated. No trace of dibasic acids or of neutral material could be found.

*Determination of Chlorine Number* (7, 8)—1 gm. of methylphthioamide, 2 gm. of phosphorus pentachloride, and 5 cc. of benzene were heated slowly in a small distilling flask to 105°. Evolution of hydrochloric acid was at first very vigorous. When the reaction ceased, the solvent and volatile products were removed as much as possible *in vacuo*. The process was repeated three times, after which the reaction product was extracted with ether. The ethereal solution was washed with water, decolorized with norit, filtered, and evaporated to dryness. The residue, a thick oil, was refluxed for 1 hour with 5 cc. of alcohol and 10 cc

of water, after which it was extracted with ether, dried, and analyzed. The substance contained 10.93 per cent of chlorine, which gives a chlorine number of 141. The phosphorus pentachloride treatment was repeated and the chlorine content rose to 16 per cent, giving a chlorine number of 220. In a separate experiment the chlorination was conducted at a temperature of 135°. The reaction product was somewhat resinous, terpene-like in odor, and had a chlorine number of 480. The results indicate that ambiguous side reactions occurred and that in the case of phthioic acid the chlorine number cannot be relied upon as a criterion of  $\alpha$  substitution.

*Stepwise Oxidation of Phthioic Acid*—A Grignard reagent was prepared from 0.5 gm. of magnesium with a requisite amount of methyl iodide in 25 cc. of ether and 2 gm. of methylphthioate were added. The mixture was refluxed for 3 hours, decomposed in the usual way, and the product was saponified so that any unchanged ester would be removed. The solution was extracted with ether. On evaporation of the ether an oil was obtained which weighed 1.25 gm. The carbinol was dehydrated at 170° with a trace of iodine and oxidized at once in 20 cc. of 95 per cent acetic acid with 0.85 gm. of chromic acid. The oxidation mixture was poured into water, extracted with ether, washed thoroughly, and separated into acid and neutral fractions. The acid portion weighed only 0.1 gm. and was not examined further. The neutral fraction, which weighed 0.8 gm., was a viscous oil. When a portion of the substance was warmed with potassium hypoiodite, it gave a soapy emulsion and the unmistakable odor of iodoform. The balance of the substance was converted into the semicarbazone in the usual way. The crude semicarbazone was a gummy solid which on solution in acetone and cooling gave a crystalline precipitate. The substance was recrystallized from acetone and from methyl alcohol. The product melted at 55–65° and was obviously a mixture from which no analytically pure substance could be isolated.

#### SUMMARY

The methyl ester of phthioic acid has been purified by fractional distillation until the specific optical rotation reached the constant value of +12.2°.

Phthioic acid prepared by saponification of the pure ester melted at 20–21°,  $[\alpha]_D = +12.56^\circ$ , and corresponded to the formula  $C_{26}H_{52}O_2$ .

Phthioamide and methylphthioamide were obtained as solid derivatives.

Phthioic acid possesses a branched chain, probably methyl groups in the  $\alpha$  position and in the neighborhood of the 11th carbon atom. Other branches exist in the chain but the number and the positions have not yet been determined.

Purified phthioic acid is biologically active, producing typical tubercular tissue when injected into normal animals.

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13. Robertson, P. W., *J. Chem. Soc.*, **115**, 1210 (1919)



# SYNTHESIS OF $\alpha$ -AMINO- $\beta$ -HYDROXY-*n*-BUTYRIC ACIDS

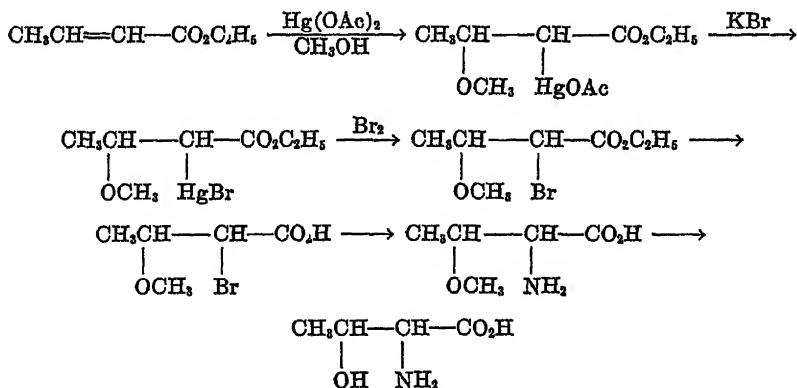
By HERBERT E. CARTER

(From the Laboratory of Physiological Chemistry, University of Illinois, Urbana)

(Received for publication, September 3, 1935)

The proof afforded by McCoy, Meyer, and Rose (1) that the essential amino acid present in their active fractions was  $\alpha$ -amino- $\beta$ -hydroxy-*n*-butyric acid has made it desirable to prepare that amino acid synthetically and test the activity of the substance obtained. It was immediately apparent that the synthesis of a physiologically active compound might be attended with considerable difficulty inasmuch as the presence of 2 unlike asymmetric carbon atoms in the molecule caused the existence of two epimeric racemic forms or four optically active isomers. Of these it seemed probable that one, or possibly two, would replace the naturally occurring form.

Abderhalden and Heyns (2) prepared  $\alpha$ -amino- $\beta$ -hydroxy-*n*-butyric acid, using the general method of Schrauth and Geller (3), which involved the following steps:



These reactions appeared to offer the best method of preparing the desired compound, since, in introducing the asymmetric

carbon atoms simultaneously by addition to a double bond, only one racemic form should be produced if no rearrangement occurred. Although Abderhalden and Heyns made no mention of the possibility of the formation of two epimers, it seemed certain from their data that the products obtained were homogeneous. In this connection it was interesting to note that the claim of Schryver and Buston (4) to the isolation of aminohydroxybutyric acid from oat glutelin was criticized by Abderhalden and Heyns on the basis of discrepancies in the melting points of derivatives of the synthetic substance and of the product of Schryver and Buston.<sup>1</sup> The possibility that these were different epimers was not considered.

The  $\alpha$ -amino- $\beta$ -hydroxy-*n*-butyric acid prepared had no growth-promoting properties even when fed in high per cent. This initial failure to obtain a physiologically active amino acid left two possibilities: that of converting the inactive form into a mixture of the two possible epimers, or that of attempting to prepare the second epimer from isocrotonic acid. The former appeared to offer the more rapid method of preparing active material synthetically.

The formyl derivative of  $\alpha$ -amino- $\beta$ -methoxy-*n*-butyric acid was prepared by the method of du Vigneaud and Meyer (5). The crude product was smoothly converted into a mixture of the two epimers by heating with sodium hydroxide and acetic anhydride. Hydrolysis with concentrated hydrobromic acid yielded a mixture of the corresponding amino acids.

The relative amounts of the two epimers produced was a matter of chance. The mixture obtained was active in 2 to 3 per cent compared with a 0.5 to 0.6 per cent requirement of the natural form. These data indicated that the approximate per cent of the desired epimer in the reaction mixture was either 40 to 50 or 20 to 25, depending on whether one or both of the enantiomorphs of the second epimer were physiologically active.

At the present time the separation and resolution of the formyl derivatives and the preparation of the active epimer from isocrotonic acid are being investigated.

<sup>1</sup> The amino acid was inactive due to the method of isolation.

## EXPERIMENTAL

*$\alpha$ -Amino- $\beta$ -Hydroxy-*n*-Butyric Acid*—This substance was prepared by the method of Abderhalden and Heyns.

*Formyl- $\alpha$ -Amino- $\beta$ -Methoxy-*n*-Butyric Acid*—26.6 gm. of  $\alpha$ -amino- $\beta$ -hydroxy-*n*-butyric acid were formylated by the method of du Vigneaud and Meyer. The yield was 20 gm. (60 per cent of the theoretical amount) of a white crystalline product melting at 173–174° after recrystallization from absolute alcohol. The formyl derivative was only slightly soluble in ethyl acetate or

TABLE I  
*Composition of Diets\**

	Diet 1	Diet 2	Diet 3
	gm	gm.	gm
Amino acid Mixture XII	23 7	23 7	23 7
Glucosamine hydrochloride ( <i>d</i> -)	1 0	1.0	1 0
Sodium bicarbonate.	0 4	0 4	0 4
Dextrin . . . . .	20.9	20 4	19 9
Sucrose.	15 0	15 0	15 0
Salt mixture†	4 0	4 0	4 0
Agar . . . . .	2 0	2 0	2 0
Lard . . . . .	26.0	26 0	26 0
Cod liver oil . . . . .	5 0	5 0	5 0
Aminohydroxybutyric acids	2 0	2 5	3 0
	100 0	100 0	100 0

\* The vitamin B factors were supplied in the form of two pills daily, each containing 75 mg of milk concentrate and 50 mg of tikitiki extract

† Osborne, T. B, and Mendel, L B, *J Biol Chem*, 37, 572 (1919).

benzene, moderately soluble in acetone and cold alcohol or cold water, highly soluble in hot alcohol or hot water.

$C_8H_{11}O_4N$  Calculated C 44 72, H 6 83, N 8 69, neutral equivalent 161  
Found. " 44 57, " 6 71, " 8 84, " " 162

*Epimerization of Formyl Derivative*—10 gm. of crude formyl derivative were dissolved in 32.8 cc of 1.895 N sodium hydroxide. 35 cc. of water were added and 70 cc. of acetic anhydride were run in slowly with shaking and warming. The mixture was heated for 15 hours at 80°. The solution was cooled and 69.1



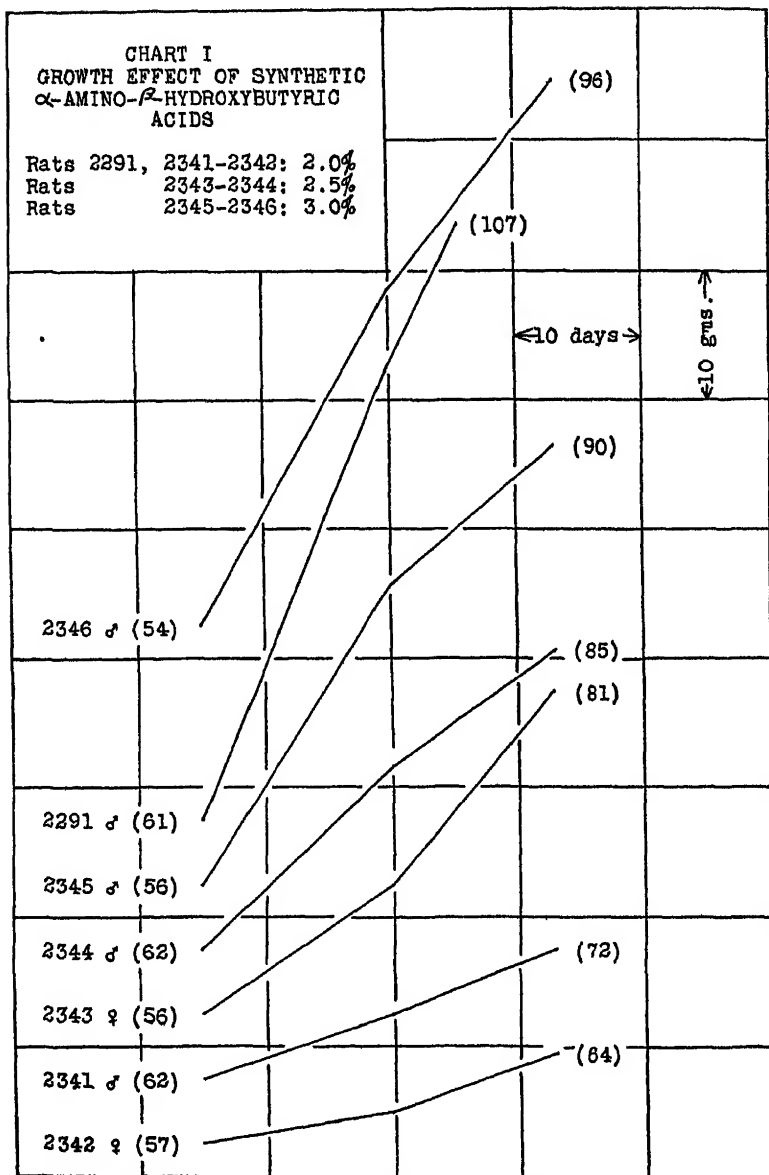


CHART I The numbers in parentheses denote the initial and final weights of the rats

cc. of 0.9 N sulfuric acid were added. The solution was concentrated *in vacuo*. Water was added and the concentration was repeated twice. Absolute alcohol was added and removed under reduced pressure. The residue was extracted with absolute alcohol and the sodium sulfate was filtered. The alcohol was removed under reduced pressure, yielding a viscous mass, which was refluxed for 2 hours with 100 cc. of 48 per cent hydrobromic acid. The resulting solution was concentrated *in vacuo*, water was added, and the solution was reconcentrated twice. The residue was dissolved in 100 cc. of water and the hydrobromic acid was removed with freshly prepared silver oxide. The excess silver was precipitated with hydrogen sulfide and the silver sulfide was filtered. The filtrate was concentrated under reduced pressure and the residue was crystallized from 80 per cent alcohol in the usual manner.

The mixture of amino acids thus prepared supported growth when included in the diet in 2 to 3 per cent. The activity of various fractions differed but slightly. Attempts to concentrate the active epimer by fractional crystallization have proved unsatisfactory as yet.

*Feeding Experiments*—The aminohydroxybutyric acid mixtures produced by the epimerization were tested for their growth-stimulating properties by incorporating them in a diet carrying all the known amino acids with the exception of hydroxyglutamic acid. The composition of the diets is shown in Table I. Amino acid Mixture XII is that described by McCoy, Meyer, and Rose. The diets contained 17.5 per cent of "effective" amino acids including glucosamine plus that supplied by the aminohydroxybutyric acids which amounted to approximately 0.5 per cent. The results of the experiments are shown in Chart I.

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## THE STRUCTURE OF *d*-XYLOMETHYLOSE

By P. A. LEVENE AND JACK COMPTON

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York)

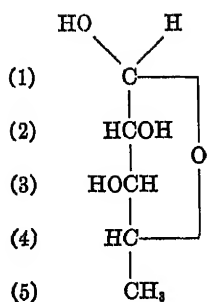
(Received for publication, October 18, 1935)

In previously assigning to *d*-xylomethylose<sup>1</sup> the structure given in Formula I, the following assumptions have been made: first, that the structure of monoacetone xylose is represented by Formula III; second, that in the unimolecular tosylation of monoacetone xylose the primary hydroxyl is preferentially esterified, and third, that the replacement of the tosyl group by iodine and subsequent reduction of the latter to a desoxy derivative cause no fundamental structural changes. The work of Haworth and Porter<sup>2</sup> establishes conclusively that Formula III represents the structure of monoacetone xylose. The second and the third assumptions have been based on the experience previously noted in the case of certain hexoses. In the present communication, experimental evidence is presented which demonstrates the validity of the above assumptions and which therefore definitely establishes the structure (I) assigned by us to *d*-xylomethylose.

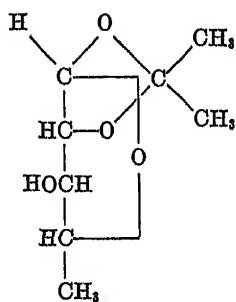
The problem of determining the structure of "*d*-xylomethylose" resolves itself into the correct allocation of the desoxy group. Since the acetone rest in monoacetone xylomethylose unquestionably occupies positions (1) and (2), the only remaining positions which the desoxy group may occupy are (3), (4), and (5). Position (4) must be considered, since the possibility of a ring shift is not excluded *a priori*. In assigning the desoxy group to position (5), the following lines of evidence are presented. First, nitric acid oxidation of completely methylated "xylomethylose" (Formulas IV and V) yields exclusively dimethyl *d*-tartaric acid (Formula VI). This result can only be explained when the desoxy

<sup>1</sup> Levene, P. A., and Compton, J., *J. Biol. Chem.*, **111**, 325 (1935)

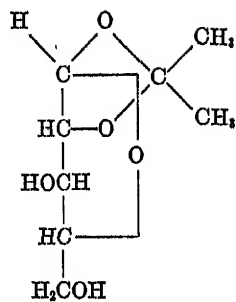
<sup>2</sup> Haworth, W. N., and Porter, C. R., *J. Chem. Soc.*, 611 (1928).



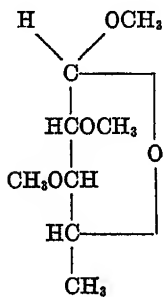
I

*d*-Xylomethylose

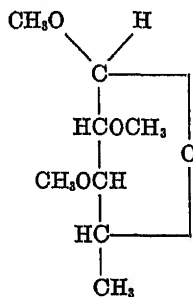
II

Monoacetone  
*d*-xylomethylose

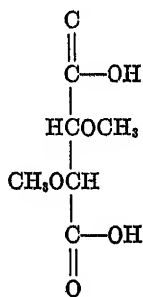
III

Monoacetone *d*-xylose

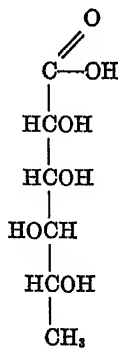
IV

2,3-Dimethyl  $\alpha$ -methyl-  
*d*-xylomethyloside

V

2,3-Dimethyl  $\beta$ -methyl-  
*d*-xylomethyloside

VI

Dimethyl  
*d*-tartaric acid

VII

*d*-Gulomethylnic acid

group is in position (5), since a desoxy group in either position (3) or (4) would lead to the formation of a dimethoxy glutaric acid.<sup>3,4,5</sup> Second, the oxidation of "*d*-xylomethylose" with silver oxide yields only silver acetate.<sup>4,6</sup> Third, the polarimetric observation of the rate of lactone formation of *d*-gulomethyloic acid<sup>7</sup> (Formula VII) indicates the formation of both  $\gamma$ - and  $\delta$ -lactones.

Completely methylated *d*-xylomethylose was obtained by the stepwise methylation of *d*-xylomethylose in a manner involving the least possible structural change. Crystalline monoacetone *d*-xylomethylose was methylated with Purdie's reagent to yield 3-methyl monoacetone *d*-xylomethylose, a colorless mobile liquid, b.p. 58–60° at 0.3 mm.,  $[\alpha]_D^{25} = -49.4^\circ$ . Acid hydrolysis of this compound yielded 3-methyl xylomethylose, isolated as a thick sirup, b.p. 100–102° at 0.8 mm.,  $[\alpha]_D^{25} = +8.1^\circ$ . Treatment of 3-methyl xylomethylose with dry methyl alcohol-hydrogen chloride (1 per cent) led to the simultaneous formation of 3-methyl  $\alpha$ -methylxylomethyloside, b.p. 58–62° at 0.3 mm.,  $[\alpha]_D^{25} = +124.5^\circ$ , and crystalline 3-methyl  $\beta$ -methylxylomethyloside, m.p. 48–50°,  $[\alpha]_D^{25} = -127.9^\circ$ . Further methylation of the  $\alpha$  and  $\beta$  isomers with Purdie's reagent led to the formation of crystalline 2,3-dimethyl  $\alpha$ -methylxylomethyloside (Formula IV), m.p. 34–35°,  $[\alpha]_D^{20} = +154.0^\circ$ , and 2,3-dimethyl  $\beta$ -methylxylomethyloside (Formula V), b.p. 38–40° at 0.5 mm.,  $[\alpha]_D^{20} = -102.4^\circ$ . Oxidation of the completely methylated  $\alpha, \beta$  pair with concentrated nitric acid yielded exclusively in each case dimethyl *d*-tartaric acid (Formula VI), identified in the case of the  $\alpha$  isomer oxidation as the free acid (m.p. 151–152°) and in the case of the  $\beta$  isomer as the dimethylamide derivative (m.p. 205–206°).

#### EXPERIMENTAL

*3-Methyl Monoacetone d*-Xylomethylose—Monoacetone *d*-xylomethylose (7.0 gm.) was dissolved in 70 cc. of methyl iodide and 40 gm. of silver oxide were added in small portions over a period of 4 hours with stirring at 50°. The mixture was then filtered and

<sup>3</sup> Kiliani, H., *Ber. chem. Ges.*, **38**, 4040 (1905).

<sup>4</sup> Micheel, F., *Ber. chem. Ges.*, **63**, 347 (1930).

<sup>5</sup> Elderfield, R. C., *J. Biol. Chem.*, **111**, 527 (1935).

<sup>6</sup> Kiliani, H., *Ber. chem. Ges.*, **32**, 2197 (1899).

<sup>7</sup> Levene, P. A., and Compton, J., *J. Biol. Chem.*, **111**, 335 (1935).

the silver residues thoroughly extracted with hot acetone. The thick sirup, obtained after concentrating the combined filtrates under diminished pressure, distilled completely under high vacuum. B. p. 58–60° at 0.3 mm. Yield 6.2 gm.  $n_D^{22}$  1.4377. The specific rotation in water was

$$[\alpha]_D^{25} = \frac{-2.86^\circ \times 100}{2 \times 2.892} = -49.4^\circ$$

The composition agreed with that of a methyl monoacetone pentomethylose.

5.422 mg. substance. 11.430 mg. CO<sub>2</sub> and 4.168 mg. H<sub>2</sub>O

5.380 " " : 6.702 " AgI

C<sub>9</sub>H<sub>18</sub>O<sub>4</sub>. Calculated. C 57.40, H 8.57, OCH<sub>3</sub> 16.48

188.1 Found. " 57.48, " 8.60, " 16.43

The substance is soluble in all the usual organic solvents and in water.

*3-Methyl d-Xylomethylose*—3-Methyl monoacetone *d*-xylomethylose (5.2 gm.) was dissolved in 100 cc. of 1 per cent sulfuric acid and heated on a boiling water bath for 60 minutes. The initial rotation of  $[\alpha]_D^{25} = -48.0^\circ$  had changed at the end of this time to the constant value  $[\alpha]_D^{25} = +8.8^\circ$ . The sulfuric acid was then exactly neutralized with barium hydroxide and the solution treated with charcoal and filtered. The clear filtrate was concentrated under diminished pressure to a sirup which was dried by the repeated addition of absolute ethyl alcohol, followed by dry benzene. The resulting sirup was dissolved in dry ether and dried over anhydrous sodium sulfate. After filtering, the ether solution was concentrated under diminished pressure to a sirup which distilled completely under high vacuum. B. p. 100–102° at 0.8 mm. Yield 3.1 gm. The specific rotation in water was

$$[\alpha]_D^{25} = \frac{+0.32^\circ \times 100}{1 \times 3.936} = +8.1^\circ$$

remaining constant. The composition agreed with that of a methyl pentomethylose.

4.900 mg. substance: 8.695 mg. CO<sub>2</sub> and 3.690 mg. H<sub>2</sub>O

4.900 " " : 7.740 " AgI

C<sub>6</sub>H<sub>12</sub>O<sub>4</sub>. Calculated. C 48.61, H 8.17, OCH<sub>3</sub> 20.93

148.1 Found. " 48.39, " 8.42, " 20.64

The substance reduced Fehling's solution strongly when heated. It is soluble in organic solvents and in water.

*3-Methyl d-Xylomethylose Phenyllosazone*—3-Methyl xylomethylose (0.15 gm.) was dissolved in 20 cc. of water and 0.5 gm. of phenylhydrazine dissolved in 3 cc. of glacial acetic acid were added. After heating at 100° for 10 minutes the osazone began to separate and upon cooling the solution, it crystallized as yellow flocks. After filtering, the product was recrystallized from 50 per cent methyl alcohol to give the constant melting point, 128–130°. The composition agreed with that of a methyl pentomethylose phenyllosazone.

4.840 mg. substance: 11.750 mg. CO<sub>2</sub> and 2.980 mg. H<sub>2</sub>O

7.350 " " : 5.260 " AgI

C<sub>18</sub>H<sub>22</sub>O<sub>2</sub>N<sub>4</sub>. Calculated. C 66.22, H 6.79, OCH<sub>3</sub> 9.50  
326.18 Found. " 66.20, " 6.88, " 9.44

*3-Methyl α- and β-Methylxylomethylosides*—3-Methyl d-xylo-methylose (2.4 gm.) was dissolved in 50 cc. of absolute methyl alcohol containing 1 per cent of dry hydrogen chloride and allowed to reflux on the steam bath for 1 hour. The initial specific rotation of  $[\alpha]_D^{25} = +10.2^\circ$  had changed at the end of this time to the constant value of  $[\alpha]_D^{25} = +5.1^\circ$ . The acid was now removed with excess silver carbonate and the resulting clear filtrate concentrated under diminished pressure to a clear mobile sirup which distilled in two fractions under high vacuum. The first fraction was the pure α isomer, b.p. 58–62° at 0.3 mm. Yield 0.9 gm.  $n_D^{25}$  1.4410. The second fraction was the pure β isomer, b.p. 72–75° at 0.3 mm., which crystallized spontaneously during the distillation. M.p. 48–50°. Yield 1.0 gm. The high vapor pressure of the α isomer makes the separation from the β isomer quite easy. The specific rotation of 3-methyl α-methylxylomethyloside in water was

$$[\alpha]_D^{25} = \frac{+3.75^\circ \times 100}{1 \times 3.012} = +124.5^\circ$$

and that of 3-methyl β-methylxylomethyloside in water was

$$[\alpha]_D^{25} = \frac{-4.20^\circ \times 100}{1 \times 3.284} = -127.9^\circ$$



The composition of both substances agreed with that of an  $\alpha$ ,  $\beta$  pair of a methyl methylpentomethyloside.

6 112 mg.	$\alpha$ isomer:	11.585 mg.	CO <sub>2</sub> and 4.780 mg.	H <sub>2</sub> O
3.615	" "	"	: 10 485	" AgI
5.232	" $\beta$	"	: 9 950	" CO <sub>2</sub> and 4.070 mg.
6.320	" "	"	: 18 315	" AgI
	C <sub>7</sub> H <sub>14</sub> O <sub>4</sub> .	Calculated.	C 51.81, H 8 70, OCH <sub>3</sub> 38.27	
162.11	Found.			
	$\alpha$ isomer.	" 51.69, "	8.75, "	38.28
	$\beta$ "	" 51 86, "	8.70, "	38.25

Neither the  $\alpha$  nor  $\beta$  isomer reduced boiling Fehling's solution but after acid hydrolysis both gave a strong test. The isomeric substances are soluble in organic solvents and in water.

*2,3-Dimethyl  $\alpha$ -Methylxylomethyloside*—3-Methyl  $\alpha$ -methylxylomethyloside (0.8 gm.) was methylated with methyl iodide (12 cc.) in the presence of silver oxide (10 gm.) in the manner previously described. Distillation of the sirupy liquid under high vacuum showed that the substance was homogeneous. B.p. 39–41° at 0.5 mm. The product crystallized completely after distillation. M.p. 34–35°. Yield 0.7 gm. The specific rotation in water was

$$[\alpha]_D^{25} = \frac{+1.83^\circ \times 100}{1 \times 1.188} = +154.0^\circ$$

The composition of the substance agreed with that of a dimethyl methylpentomethyloside.

4 400 mg.	substance.	8 795 mg.	CO <sub>2</sub> and 3.560 mg.	H <sub>2</sub> O
3.690	" "	"	: 14.720	" AgI
	C <sub>8</sub> H <sub>16</sub> O <sub>4</sub> .	Calculated.	C 54.50, H 9.15, OCH <sub>3</sub> 52 80	
176.13	Found.	" 54.51, "	9.05, "	52.65

*Preparation of Dimethyl *d*-Tartaric Acid from 2,3-Dimethyl  $\alpha$ -Methylxylomethyloside*—0.4 gm. of 2,3-dimethyl  $\alpha$ -methylxylomethyloside (m.p. 34–35°) was dissolved in 5 cc. of concentrated nitric acid (1.42) and placed in a water bath at 50°. The temperature was gradually raised over a period of 30 minutes to 100°, after which it was maintained at 95–100° for 7 hours. At the end of this time the solution was concentrated under diminished pressure to a solid crystalline mass at 40°. The last traces of nitric acid were then removed under high vacuum by placing

the crystalline material in a vacuum desiccator over solid sodium hydroxide for 3 hours. The crude product was then dissolved in dry ether and filtered. The clear filtrate, upon concentrating under diminished pressure, crystallized completely and was filtered off and washed several times with dry ether. The melting point of the purified material was 151–152°, which was unchanged when mixed with an authentic specimen of dimethyl *d*-tartaric acid.<sup>8</sup> Yield 0.3 gm. The specific rotation in water was

$$[\alpha]_D^{25} = \frac{+1.43^\circ \times 100}{1 \times 1.992} = +71.8^\circ$$

*2,3-Dimethyl β-Methylxylomethyloside*—3-Methyl β-methylxylomethyloside (0.8 gm.) was methylated with methyl iodide (12 cc.) in the presence of silver oxide (10 gm.) in the usual manner. The mobile sirup thus obtained distilled completely under high vacuum, b.p. 38–40° at 0.5 mm.  $n_D^{25}$  1.4261. Yield 0.6 gm. The specific rotation in water was

$$[\alpha]_D^{25} = \frac{-1.91^\circ \times 100}{1 \times 1.866} = -102.4^\circ$$

The composition agreed with that of a dimethyl methylxylomethyloside.

5.380 mg. substance:	10.735 mg. CO <sub>2</sub>	and 4.310 mg. H <sub>2</sub> O
4.592 " " "	18.240 " AgI	
C <sub>8</sub> H <sub>16</sub> O <sub>4</sub> .	Calculated.	C 54.50, H 9.15, OCH <sub>3</sub> 52.80
176.13	Found.	" 54.51, " 8.96, " 52.21

*Preparation of d-Dimethoxysuccinomethylamide from 2,3-Dimethyl β-Methylxylomethyloside*—The oxidation of 2,3-dimethyl β-methylxylomethyloside (0.4 gm.) with concentrated nitric acid (5 cc.) to yield dimethyl *d*-tartaric acid was carried out as described above for the α isomer. In this case the reaction product was esterified by refluxing for 6 hours with absolute methyl alcohol (20 cc.) containing 2 per cent dry hydrogen chloride. At the end of this time the acid was neutralized with excess silver carbonate and the solution filtered. The sirup obtained upon concentrating the filtrate under diminished pressure was dissolved in dry ether,

<sup>8</sup> Purdie, T. P., and Irvine, J. C., *J. Chem. Soc.*, 79, 959 (1901).

dried over anhydrous sodium sulfate, filtered, the solvent evaporated, and the product distilled under high vacuum. B.p. 78–80° at 0.3 mm. Yield 0.5 gm.

The material thus obtained was dissolved in 5 cc. of absolute methyl alcohol, cooled to 0°, and saturated with dry methylamine. After standing for 1 day at 0° the solution was concentrated under diminished pressure, whereupon the amide crystallized completely and was purified by recrystallizing from ethyl acetate. M.p. 205–206°. A mixed melting point of this material with an authentic specimen of *d*-dimethoxysuccinomethylamide<sup>9</sup> (m.p. 205–206°) showed no depression.

*Oxidation of d-Xylomethylose with Silver Oxide*—*d*-Xylomethylose (1.0 gm.) was dissolved in 50 cc. of water containing a suspension

TABLE I  
Rate of Lactone Formation of *d*-Gulomethylonic Acid

Time	$[\alpha]_D^{25}$	Time	$[\alpha]_D^{25}$
min	degrees	hrs	degrees
8	–5.2	20	–2.5
40	–2.7	48	–10.6
60	–0.9	96	–24.6
120	+1.8	288	–42.2
220	+4.0	20 (Days)	–47.9

of 12 gm. of silver oxide. The mixture was heated on the water bath for 8 hours at 80° with occasional stirring. The solution was then filtered and the filtrate concentrated under diminished pressure to 5 cc., whereupon a large quantity of crystalline material separated. After removal by filtration, the substance was recrystallized from water and dried over calcium chloride. The composition of the substance agreed with that of silver acetate.

13.800 mg. substance. 8.900 mg. Ag

$C_5H_8O_3Ag$ . Calculated. Ag 64.6  
166.88 Found. " 64.4

*Lactone Formation of d-Gulomethylonic Acid*—*d*-Gulomethylonolactone (0.3005 gm.) was dissolved in exactly 5.0 cc. of 0.429 N

<sup>9</sup> Haworth, W. N., and Jones, D. I., *J. Chem. Soc.*, 2349 (1927)

sodium hydroxide solution and allowed to stand 1 hour at room temperature. The specific rotation,  $[\alpha]_D^{25} = +9.7^\circ$  (calculated as sodium gulomethylonate), remained constant at the end of this time. To 2.5 cc. of this solution there were then added 2.05 cc. of 0.498 N hydrochloric acid and the resulting solution was diluted to exactly 5.0 cc. The initial specific rotation was observed as soon as possible and the change in rotation observed at various time intervals, as shown in Table I.



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